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THE WISTAR INSTITUTE OF ANATOMY AND BIOLOGY

WOODLAND AVENUE AND THIRTY-SIXTH STREET, PHILADELPHIA 4, PA.

THE EFFECT OF VARIATION IN FILTRATION RATE ON THE URINARY CONCENTRATING MECHANISM IN THE SEAL, PHOCA VITULINA L.

LOT B. PAGE, J. C. SCOTT-BAKER,¹ G. A. ZAK,² E. L. BECKER
AND C. F. BAXTER³

*Department of Physiology, New York University College of Medicine, New York
and the Mt. Desert Island Biological Laboratory, Salisbury Cove, Maine*

TWO FIGURES

In man, the maximal attainable osmotic U/P ratio is 3.6 to 4.2 (Smith, '51). This degree of concentration can only be achieved when the quantity of excreted solute is very small (Gamble, Butler, '44; Smith, '51). As the quantity of solute claiming excretion is increased by solute loading, the maximal attainable osmotic U/P ratio decreases and approaches 1.0 as a limiting asymptote (Hervey, McCance and Tayler, '46; Rapoport, Brodsky, West and Mackler, '49). It has been shown that during substantial osmotic diuresis the osmotic concentration of the urine is not influenced by the chemical or ionic nature of the contained solutes, but depends on the total quantity of osmotically active material destined for excretion (Hervey et al., '46; Rapoport et al., '49).

Recent studies on the nature of the concentrating mechanism (Wesson and Anslow, '52; Ladd, '52; Page and Reem, '52) have indicated that hypertonic urine may be elaborated by the operation of a reabsorptive process which is independent of facultative (distal) water reabsorption and which removes a small quantity of water, limited in rate, from an isosmotic

¹ Fulbright Fellow, London.

² Fellow of the New York Heart Association.

³ Research Fellow U. S. Army Shock Project.

tubular fluid. This process (the concentrating mechanism) has been termed $T^c_{H_2O}$ by Wesson and Anslow ('52).

In the harbor seal, *Phoca vitulina*, Smith ('36) reported freezing point depressions of the urine ranging as low as $\Delta = -3.988^\circ\text{C}$. in a fasting animal. Assuming a plasma osmolarity of 330 mosm/liter, this would represent an osmotic U/P ratio of 6.5. The urinary concentrating mechanism thus appears to be highly developed in this species. In the seal, the filtration rate may be markedly elevated by feeding (Hiatt and Hiatt, '42) and greatly reduced by excitation of the diving reflex (Bradley and Bing, '42). This species therefore affords an opportunity to study the effect on the concentrating mechanism of wide variation in filtration rate during solute loading.

METHODS

The animals used in the study consisted of 2 male and 4 female seals, captured shortly after weaning and ranging in weight from 17 to 24 kg.⁴ They were kept in open cages partially immersed in sea water and were fed with fresh herring. Supplementary multivitamin therapy was given by intramuscular injection two to three times a week, and intramuscular injections of penicillin were given following each experiment to prevent the development of cutaneous abscesses at venepuncture sites. At first, attempts were made to produce osmotic diuresis by means of urea and hypertonic mannitol solutions. However, death resulted early in the experiment in all cases.⁵ It therefore became necessary to carry out the experiment with saline diuresis which the animals could tolerate without ill effects.

During the experiment the seal was strapped on an animal board. Infusions were given by a constant infusion pump through a plastic catheter lying in a flipper vein. After a 30-

⁴ We are indebted to Mr. P. Goggins, Fisheries Research Station, Boothbay Harbor, Maine, for supplying the animals used in this study.

⁵ After administering intravenous urea solutions in isotonic saline, death apparently resulted from massive hemolysis. Following intravenous mannitol solutions, death was attended by nystagmus and convulsions.

minute equilibration period, during which creatinine solution was infused at $1.5 \text{ cm}^3/\text{minute}$, 1000 to 1800 cm^3 of 0.9% NaCl solution were administered at a rate of $40 \text{ cm}^3/\text{minute}$, and urine collections were taken at 10- to 20-minute intervals during the ensuing diuresis. Eighty to $150 \text{ mU}/\text{hour}$ of Pitressin were administered throughout the experiment either by constant infusion or by repeated intravenous injections, to assure maximal osmotic concentration. In view of Ladd's reports ('51) that in man copious excretion of sodium and water is induced if subjects are prehydrated by the ingestion of large amounts of water 8 to 13 hours before infusing NaCl solution, attempts were made in three experiments to reproduce this phenomenon in the seal. In such experiments, 1000 to 1500 cm^3 of tap water were administered by stomach tube 8 to 13 hours before the experiment. In 7 experiments 40 to 80 mg of Thiomerin (Campbell Pharmaceutical Company, New York) were administered intravenously at the beginning of the experiment, in an attempt to induce an increased excretion of sodium. In 4 additional experiments, both prehydration and Thiomerin were used.

Urine collection was obtained in females by means of an indwelling bladder catheter. The males, which could not be catheterized, were placed in a prone position with the urinary papilla lying over a funnel through which they voided into graduated cylinders at 10- to 20-minute intervals. The bladder was not rinsed with distilled water because of the uncertainty of the diluting effect on the osmotic activity of the urine, as determined by freezing point depression. Venous blood was drawn at appropriate intervals into syringes moistened with heparin.

To determine the osmotic U/P ratio during oliguria, bladder urine and heparinized venous blood were collected after fasting the animals for 12 to 18 hours. Freezing point depressions of the urine and plasma samples were determined as described below and the osmotic U/P ratios calculated from these data.

The diving reflex was elicited by closing the nostrils with a cone placed over the snout. Periods of "diving" were interrupted from time to time to permit the animal to breathe.

The filtration rate was measured by the exogenous creatinine clearance (Smith, '36), creatinine being determined by the method of Bonsnes and Taussky ('45). The freezing point depression of urine and plasma was determined by the method described by Wesson ('52) using a Leeds and Northrup Wheatstone Bridge and a Western Electric Thermistor, no. 14B. Sodium and potassium were determined with a Perkin-Elmer flame spectrophotometer, model 52A, using a propane flame. Urea was determined by the hypobromite method of Van Slyke and Kugel ('33), chloride by iodometric titration (Van Slyke and Hiller, '47), and plasma protein by the copper sulfate method of Van Slyke et al. ('50).

The osmolarity of plasma and urine was calculated as freezing point depression in degrees centigrade divided by 1.86, the molal freezing point depression of an ideal solute. Because the plasma protein concentration varied during the experiment, the filtration rate of water is calculated as wC_F , where $w=1$ — the plasma protein concentration in gm/cm^3 .

RESULTS

The freezing point depression of plasma in fasting animals ranged from 0.545 to 0.614°C., equivalent to 293 to 340 mosm/l, and averaging 330 mosm/l.

To approximate the maximal osmotic U/P ratio during oliguria this value was determined 9 times in 4 seals after fasting the animals for 12 to 18 hours. The values ranged from 4.67 to 5.96 and averaged 5.60.

Saline diuresis experiments were carried out 6 times in one female (seal no. 1) and 7 times in one male (seal no. 2). Single experiments in each of 4 other seals gave similar results and will not be reported in detail. Results of typical diving experiment are shown in figure 1 and table 1. All values are expressed in terms of kidney weight. In the data on seal no. 1, the filtration rate varied from 11.8 to 128 $\text{cm}^3/\text{minute}$ per 100 gm kidney weight. The lowest values were obtained during excitation of the diving reflex, and the highest values 2 to 4 hours

after a fish meal. Diving experiments were not carried out in males because they could not be induced to void during the period of low urine flow. In the data on seal no. 2, therefore, the lowest filtration rates were obtained after a 12- to 18-hour fast. In this animal, the filtration rate varied from 50 to 121 $\text{cm}^3/\text{minute}$ per 100 gm kidney weight. With saline diuresis, it proved impossible to induce excretion rates of solute greater

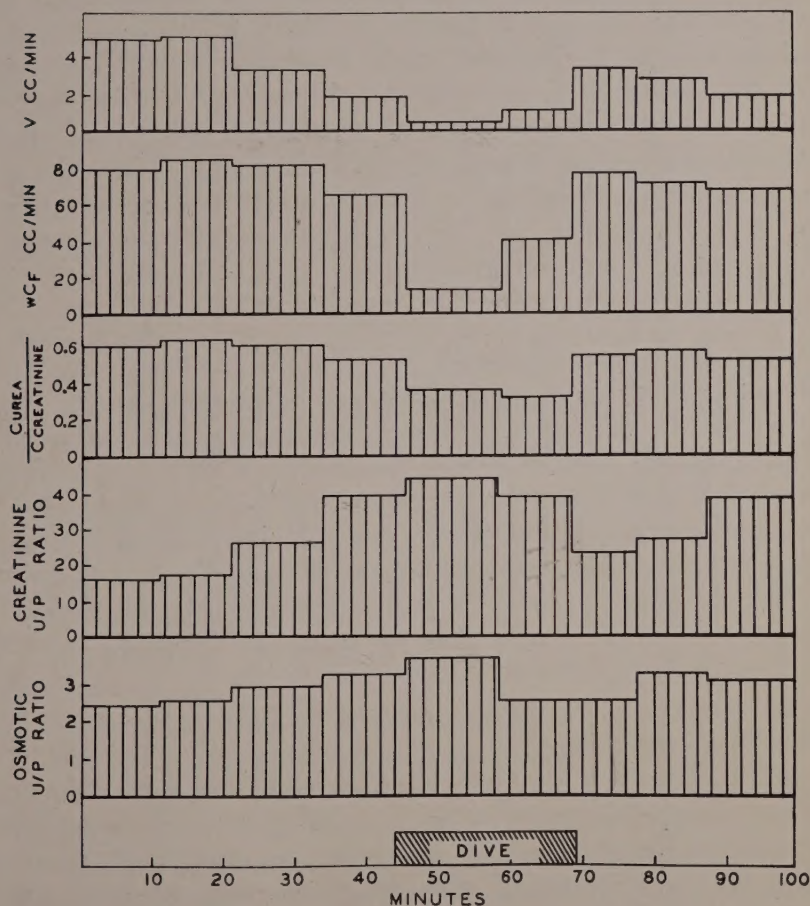


Fig. 1 Variations in renal function during excitation of the diving reflex. Diuresis had been established before zero time by administering 40 mg of Thiomerin and the infusion of 0.9% NaCl at 40 $\text{cm}^3/\text{minute}$. The data are corrected to 100 gm kidney weight. Further data from this experiment are given in table 1.

TABLE 1
Osmotic diuresis in the seal

Seal no. 1, ♀

Thimerin and saline diuresis was induced before period 1; the diving reflex was elicited during periods 5 and 6. All values are calculated per 100 gm kidney weight

PERIOD	V cm^3/min	wC_F cm^3/min	U_{osm} mos/l	OSMOTIC $\frac{U/P}{\text{RATIO}}$	$\frac{U_{\text{osm}} V}{P_{\text{osm}}}$ cm^3/min	$T^c_{\text{H}_2\text{O}}$ cm^3/min	$\frac{U_{\text{osm}} V \times 100}{P_{\text{osm}} \text{wC}_F}$ %	$\frac{V \times 100}{\text{wC}_F}$ %	$\frac{C_{\text{area}}}{C_{\text{creatinine}}}$	CREATININE $\frac{U/P}{\text{RATIO}}$
1	5.1	80.2	734	2.55	13.1	8.0	16.3	6.4	0.62	16.7
2	5.2	86.1	750	2.66	13.9	8.7	16.1	6.1	0.65	17.4
3	3.3	82.7	841	3.04	9.9	6.7	12.0	4.0	0.63	26.6
4	1.8	65.8	919	3.34	5.8	4.1	8.9	2.7	0.54	39.6
5 ¹	0.28	11.8	1053	3.82	1.07	0.8	9.1	2.4	0.37	44.4
6 ¹	1.1	39.9	717	2.61	2.8	1.8	7.1	2.7	0.33	39.1
7	3.4	76.5	712	2.59	8.9	5.5	11.7	4.5	0.56	23.6
8	2.8	72.0	925	3.38	9.6	6.8	13.3	4.0	0.60	27.0
9	1.9	68.3	860	3.17	5.9	4.1	8.7	2.7	0.54	38.8

¹ Diving.

than 16 to 18% of the filtered load. These high excretion rates were obtained with a combination of "prehydration" and Thiomerin. However, our data do not afford any evidence regarding the effect of either prehydration or Thiomerin on the excretion of NaCl as such.

As in man (Hervey et al., '46; Rapoport et al., '49) the osmotic U/P ratio declined toward 1.0 as the urine flow increased during osmotic diuresis. These variations in osmotic U/P ratio are poorly correlated with filtration rate, but are well correlated with variations in the quantity of solute excreted, expressed as the osmolar clearance (C_{osm}). This value was calculated from the osmolarity of plasma and urine as determined by freezing point depression, and represents the isosmotic equivalent, in $\text{cm}^3/\text{minute}$, of all the osmotically active material excreted in the urine.

$$C_{osm} = \frac{U_{osm} V}{P_{osm}} \quad (1)$$

Since physiological doses of Pitressin were given in these experiments, the urine was always hypertonic and the osmolar clearance was greater than the urine flow. The difference between C_{osm} and V represents, in $\text{cm}^3/\text{minute}$, the quantity of water which would have to be reabsorbed from an isosmotic tubular fluid to produce the degree of osmotic concentration observed in the urine. This quantity has been called $T^c_{H_2O}$ by Wesson and Anslow ('52):

$$T^c_{H_2O} = \frac{U_{osm} V}{P_{osm}} - V \quad (2)$$

In figure 2, $T^c_{H_2O}$ is plotted against C_{osm} . At the lowest values of C_{osm} , the data fall on or near the linear regression line representing the average oliguric osmotic U/P ratio of 5.6; at higher values of C_{osm} , the osmotic U/P ratio decreases, and the data on both animals progressively deviate from this line. A constant (or maximal) value of $T^c_{H_2O}$ was not observed in this study, although in the data on seal no. 1, $T^c_{H_2O}$ appears to be approaching a constant value at the highest osmolar clearances attained.

DISCUSSION

It has recently been suggested (Wesson and Anslow, '52; Smith, '52) that water reabsorption by the kidney is partitioned into three moieties: a passive, isosmotic proximal process (obligatory water reabsorption); a passive, isosmotic distal process, activated by the antidiuretic hormone (facultative water reabsorption); and the reabsorption of a small quantity of water without solute from a volume of tubular urine ordinarily grossly diminished in bulk by the proximal and distal processes (concentrating mechanism).

Accepting the hypothesis that obligatory and facultative water reabsorption are isosmotic processes under maximal ADH activation, the quantity of tubular fluid presented to the concentrating mechanism is C_{osm} . The work of Chesley ('38) indicates that when C_{osm} is small, an osmotic gradient imposes a limitation on $T^c_{H_2O}$, the quantity of water reabsorbed by the concentrating mechanism. In the seal, this osmotic ceiling is approximated by the averaged oliguric osmotic U/P ratio of 5.6. This figure is substantially higher than the maximum osmotic U/P ratio in man (3.6 to 4.2) (Smith, '51) or the dog (4.5) (Page and Reem, '52).

Recent studies of the concentrating mechanism have indicated that when C_{osm} is increased $T^c_{H_2O}$ is limited by a maximal rate. In a study of saline diuresis in prehydrated human subjects, receiving a constant infusion of Pitressin to maintain osmotic concentration, Ladd ('52) concluded that urine of variable hypertonicity with respect to sodium is elaborated by the removal of an essentially constant quantity of water from an isosmotic tubular fluid at all values of $V/wC_F \times 100$ above 3.0. This figure in Ladd's data is $2.0 \text{ cm}^3/100 \text{ cm}^3$ of glomerular filtrate. In the dog, Page and Reem ('52) have shown that when C_{osm} increases to values above $14\text{--}16 \text{ cm}^3/\text{minute}$ per 100 gm kidney weight, $T^c_{H_2O}$ reaches approximately constant maximal values of 3.8 and $5.4 \text{ cm}^3/\text{minute}$ per 100 gm kidney weight, respectively, in the two dogs studied. It was not possible in either man or dog to vary the filtration rate over a sufficiently

wide range to determine whether $T^c_{H_2O}$ is functionally related to the filtration rate, or limited by an absolute rate in $\text{cm}^3/\text{minute}$. However, in the dog it appeared that the filtration rate provided a better denominator for correlating data from different individuals than kidney weight.

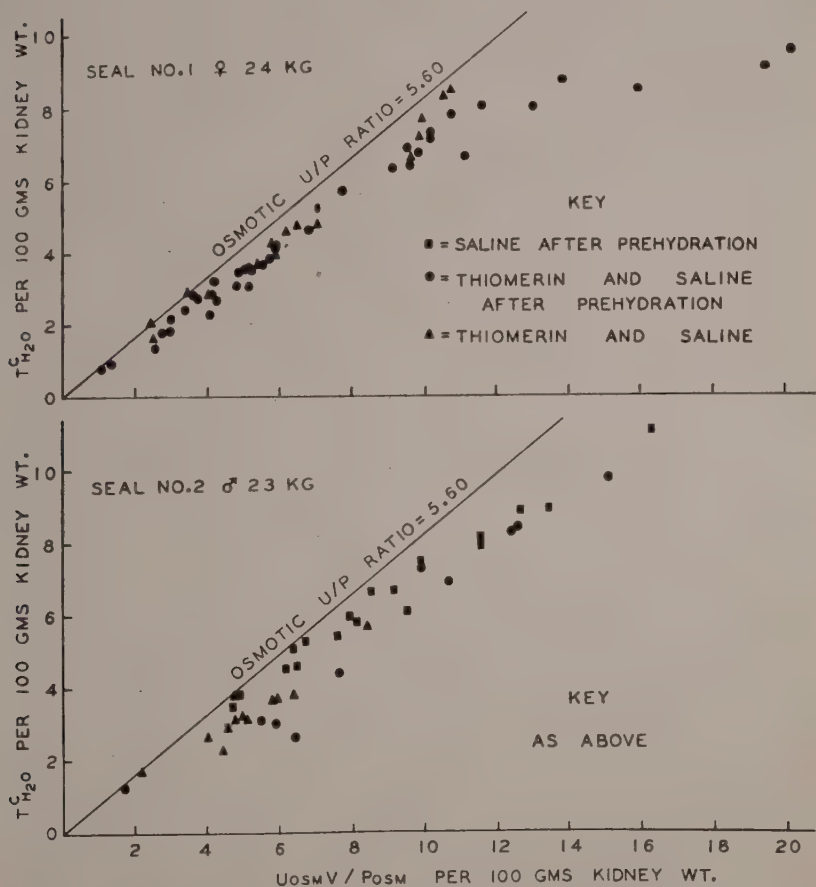


Fig. 2 Relationship between $T^c_{H_2O}$ and C_{osm} in 6 experiments on seal no. 1 (top) and 7 experiments on seal no. 2 (bottom). Each point represents one clearance period. The data are corrected to 100 gm kidney weight. Variations in filtration rate of 11.8 to 128 $\text{cm}^3/\text{minute}$ per 100 gm kidney weight in seal no. 1, and 50 to 121 $\text{cm}^3/\text{minute}$ per 100 gm kidney weight in seal no. 2, could not be correlated with variations in $T^c_{H_2O}$.

The relationship between C_{osm} and $T_{H_2O}^c$ in the seal (fig. 2) is similar to the relationship in the dog at values of C_{osm} below the point at which $T_{H_2O}^c$ becomes constant and maximal for that species. It seems probable that the basic mechanism is similar in both species, but that a maximum value for $T_{H_2O}^c$ can be reached in the seal only at higher osmolar clearances than those attained in this study. The maximal values for $T_{H_2O}^c$ in these animals must be greater than 9.8 cm³ and 11.5 cm³ per 100 gm kidney weight for seals no. 1 and no. 2, respectively, as compared to 3.8 and 5.4 cm³ in the dog, as cited above.

Since C_{osm} is the quantity of water delivered to the concentrating mechanism, C_{osm} and $T_{H_2O}^c$ are related as load and Tm are related with respect to reabsorptive mechanisms such as those for glucose and other substances. Our data indicate that there is a large splay in the "titration curve" for $T_{H_2O}^c$ in the seal. This large splay is also seen in the dog (Page and Reem, '52) but appears to be less marked in man (Zak, Brun and Smith, in preparation).

As shown in figure 2, the concentrating operation, $T_{H_2O}^c$, increases in proportion to the C_{osm} (though at a rate somewhat less than that required to give a maximal osmotic U/P ratio). This proportionality could result from the circumstance that as C_{osm} increases, a larger quantity of water is delivered to the reabsorption system, whether in a few or in many nephrons, cannot be determined. However, in view of the fact that ten-fold variations in the filtration rate in seal no. 1 and over two-fold variations in seal no. 2 did not disturb this positive correlation, we conclude that the functional relationship between load and water reabsorption is independent of the filtration rate, and by inference that the operation of the concentrating mechanism is intrinsically independent of the filtration rate.

The question arises whether variations in filtration rate associated with diving involve changes in the activity of all glomeruli, or changes in the number of active glomeruli. Hiatt and Hiatt ('42) demonstrated that glucose Tm remained con-

stant between the fasting and the postabsorptive state, though the filtration rate (and renal plasma flow) were almost doubled after feeding. We find that (during saline diuresis) at high filtration rates, the urea/creatinine clearance ratio averages 0.59 in 4 experiments, indicating reabsorption of urea, presumably by passive reabsorption as in other species, a conclusion also reached by Ladd et al. ('51) in studies of water diuresis in the seal. This ratio decreased during diving (4 experiments) to the range of 0.28–0.39, which might be interpreted as indicating that glomerular activity was reduced throughout the kidney, affording more favorable circumstances for urea reabsorption; but no definite conclusion can be drawn on this point since the creatinine U/P ratio increased during diving, and we have no knowledge of the effect of changes in the creatinine U/P ratio on the urea/creatinine ratio. The question of the distribution of glomerular activity during diving therefore remains unanswered.

SUMMARY

1. The osmotic U/P ratio during oliguria was determined 9 times in 4 seals after a 12- to 18-hour fast. Values ranged from 4.67 to 5.96 and averaged 5.60.

2. Saline diuresis, with or without Thiomerin, was induced 17 times in 6 seals. Pitressin was administered in physiological doses to maintain maximal osmotic concentration. Variations in filtration rate were induced by feeding and by eliciting the diving reflex.

3. As the quantity of excreted solute (C_{osm}) increases, the quantity of water reabsorbed by the concentrating mechanism ($T_{H_2O}^c$) increases, though at a rate less than is required to maintain a maximal osmotic U/P ratio. At the highest values of C_{osm} attained, in one seal, $T_{H_2O}^c$ appeared to reach or approach a maximal value; a maximal value was, however, not attained in the other animals.

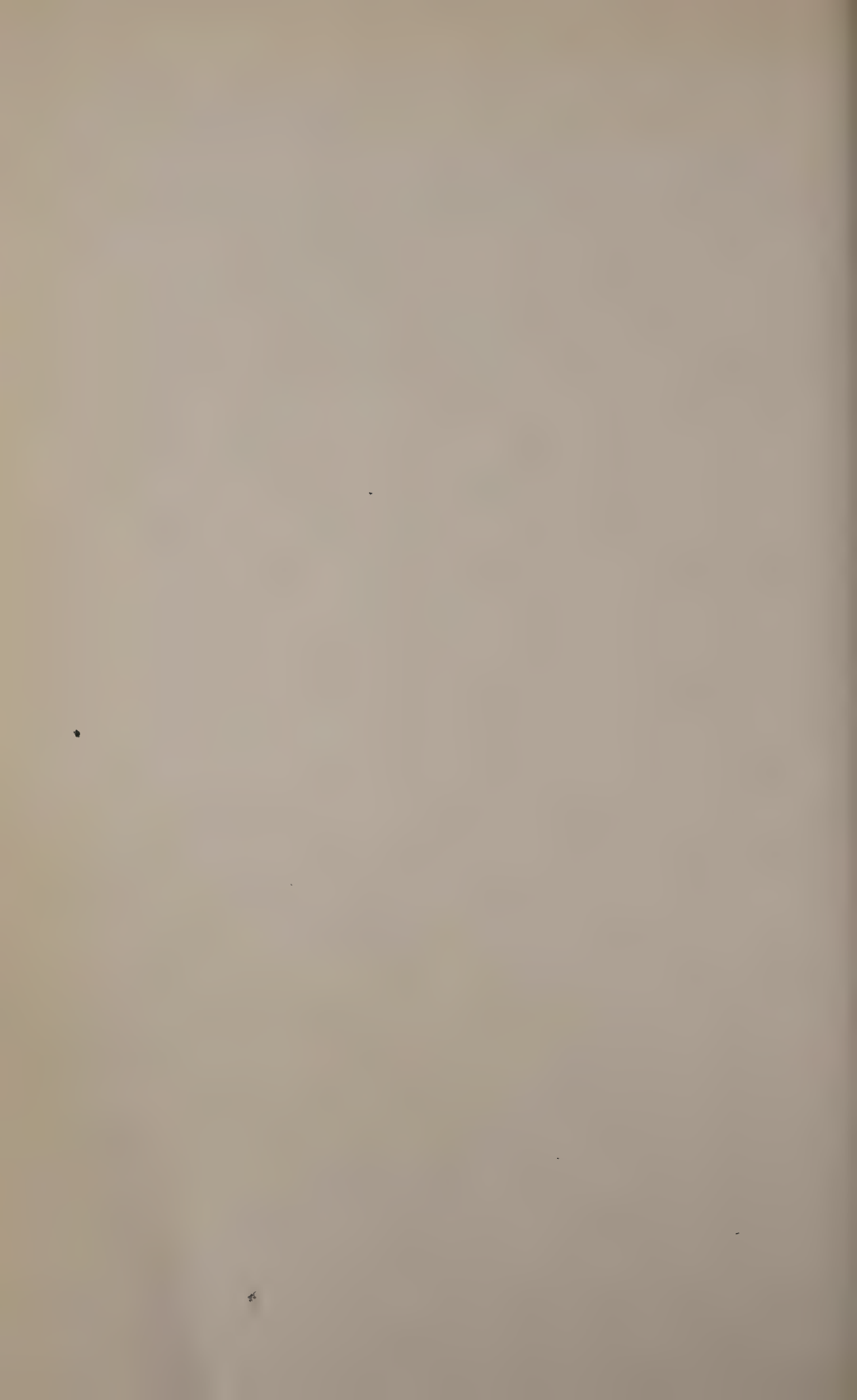
4. Variations of filtration rate of as much as tenfold fail to influence the proportional relationship between C_{osm} and

$T_{H_2O}^c$, and it is inferred that the operation of the concentrating mechanism is independent of the filtration rate.

LITERATURE CITED

- BONSNES, R. W., AND H. H. TAUSKY 1945 On the colorimetric determination of creatinine by the Jaffe reaction. *J. Biol. Chem.*, 158: 581.
- BRADLEY, S. E., AND R. J. BING 1942 Renal function in the harbor seal (*Phoca vitulina* L.) during asphyxial ischemia and progenic hyperemia. *J. Cell. and Comp. Physiol.*, 19: 229.
- CHESLEY, L. C. 1938 Renal excretion at low urine volumes and the mechanism of oliguria. *J. Clin. Invest.*, 17: 591.
- GAMBLE, J. L., AND A. M. BUTLER 1944 Measurement of the renal water requirement. *Trans. Asso. Am. Physicians*, 58: 157.
- HERVEY, G. R., R. A. MCCANCE AND R. Q. C. TAYLER 1946 Forced diuresis during hydropenia. *Nature*, 157: 338.
- HIATT, E. P., AND R. B. HIATT 1942 The effect of food on the glomerular filtration rate and renal blood flow in the harbor seal (*Phoca vitulina* L.). *J. Cell. and Comp. Physiol.*, 19: 221.
- LADD, M. 1951 Effect of prehydration on the response to saline infusion in man. *J. Appl. Physiol.*, 3: 379.
- 1951 Effect of prehydration upon renal excretion of sodium in man. *J. Appl. Physiol.*, 3: 603.
- 1952 Renal excretion of sodium and water in man as affected by prehydration, saline infusion, Pitressin and Thiomerin. *J. Appl. Physiol.*, 4: 602.
- LADD, M., L. G. RAISZ, C. H. CROWDER, JR., AND L. B. PAGE 1951 Filtration rate and water diuresis in the seal, *Phoca vitulina* L. *J. Cell. and Comp. Physiol.*, 38: 157.
- PAGE, L. B., AND G. H. REEM 1952 Urinary concentrating mechanism in the dog. *Am. J. Physiol.*, 171: 572.
- RAPOPORT, S., W. A. BRODSKY, C. D. WEST AND B. MACKLER 1949 Urinary flow and excretion of solutes during osmotic diuresis in hydropenic man. *Am. J. Physiol.*, 156: 433.
- SMITH, H. W. 1936 The composition of urine in the seal. *J. Cell. and Comp. Physiol.*, 7: 465.
- 1951 *The Kidney Structure and Function in Health and Disease*. New York, Oxford University Press.
- 1952 Renal excretion of sodium and water. *Federation Proc.*, 11: 701.
- VAN SLYKE, D. D., AND A. HILLER 1947 Application of Sendroy's iodometric chloride titration to protein-containing fluids. *J. Biol. Chem.*, 167: 107.
- VAN SLYKE, D. D., A. HILLER, R. A. PHILLIPS, P. B. HAMILTON, V. P. DOLE, R. M. ARCHIBALD AND H. H. EDER 1950 The estimation of plasma protein concentration from plasma specific gravity. *J. Biol. Chem.*, 183: 331.

- VAN SLYKE, D. D., AND V. H. KUGEL 1933 Improvements in manometric micro-Kjeldahl and blood urea methods. *J. Biol. Chem.*, 102: 489.
- WESSON, L. G. 1952 *Methods in Medical Research Vol. V*. Chicago. Year Book Publishers, Inc.
- WESSON, L. G., AND W. P. ANSLOW 1952 Effect of osmotic and mercurial diuresis on simultaneous water diuresis. *Am. J. Physiol.*, 170: 255.



ANAEROBIC NUTRITION OF SACCHAROMYCES CEREVISIAE

II. UNSATURATED FATTY ACID REQUIREMENT FOR GROWTH IN A DEFINED MEDIUM¹

A. A. ANDREASEN² AND T. J. B. STIER

Department of Physiology, Indiana University, Bloomington

ONE FIGURE

It is well known that various strains of the yeast *Saccharomyces cerevisiae* multiply to a very limited extent in media containing water soluble ingredients when the cultures are deprived of oxygen (Pasteur, 1879; A. Brown, '05; H. Brown, '14; Windisch, '32; White and Munns, '51). The scant anaerobic growth that occurs has been attributed to cellular reserves of oxygen or some oxidized material formed during previous growth in the presence of oxygen.

It has been found, however, that very good anaerobic growth is obtained when ergosterol, solubilized in the surfactant Tween 80, is employed in a defined medium (Andreasen and Stier, '53). Ergosterol, or certain other sterols, was thus shown to be essential for anaerobic growth. It was subsequently discovered, however, that the Tween 80 was supplying another essential anaerobic nutrient, oleic acid. Thus, *both* ergosterol and oleic acid are required in the defined medium in order to obtain anaerobic growth. The purpose of this paper is to present experimental evidence for the essentiality of oleic acid or other unsaturated fatty acids for anaerobic growth.

¹ This work was aided by a grant from Joseph E. Seagram & Sons, Inc.

² Seagram Research Associate at the Laboratory of Cell Physiology, Indiana University, assigned by Joseph E. Seagram & Sons, Inc., Louisville, Kentucky, February, 1950.

EXPERIMENTAL PROCEDURE

Yeast strain. All experiments were conducted with a distillery type yeast; a haploid strain of *S. cerevisiae*, SC-1 (DCL), obtained from Joseph E. Seagram and Sons, Inc., Louisville, Kentucky.

Growth criterion. The number of yeast cells in a known dilution of culture was determined by direct count in a Neubauer chamber. All buds the size of the smallest independent cell were included in the count. The populations were expressed as million (M) cells/ml. The samples were preserved by acidification and refrigeration prior to counting.

Removal of oxygen. Anaerobic conditions were obtained by thoroughly flushing all experimental vessels and media with Linde "high-purity dry nitrogen." The manufacturer claims an oxygen content of less than 20 ppm (or *ca* 0.01 mm Hg).

Defined basal media. Three defined media were employed for both the inocula and the shake cultures. These media were modifications of Difco-Yeast Nitrogen Base, which was formulated by Wickerham ('51) for the classification of yeasts. One of the preliminary media, designated Basal no. 1, differed from the commercial product in that (1) it was compounded from laboratory chemicals, (2) the phosphate concentration was increased to 0.3% and (3) the vitamins were doubled in concentration. Also, nicotinamide was arbitrarily substituted for niacin.

The second medium, designated Basal no. 2, contained for the most part only ingredients that were found to be indispensable or stimulatory. It differed from Difco-Yeast Nitrogen Base in that (1) it contained more ammonium sulfate, 0.7%, and phosphate, 0.3%, (2) the three amino acids were omitted and (3) only 5 of the vitamins were retained. The concentrations of the 5 vitamins were increased several fold as follows: biotin 8 ×, pantothenate 8 ×, nicotinamide 6 ×, thiamine 4 × and pyridoxine 4 ×.

The third, Basal no. 3, medium was employed for certain experiments because of its convenience. It consisted of Difco-

Yeast Nitrogen Base supplemented with increased concentrations of phosphate and ammonium sulfate, as indicated for the Basal no. 2 medium, and a 5-fold increase in the 5 essential vitamins.

In addition to these components, the media also contained 12% glucose and 16 volume % of 0.3 M sodium succinate buffer, pH 5.0. The final culture volume was always 25 ml.

Inoculum. Inocula for the shake cultures were taken from an anaerobic culture unit and were used in an amount to give an initial population of *ca* 0.5–0.7 M cells/ml. The anaerobic culture unit (Andreasen and Stier, '53) is an all-glass apparatus that provides a regular supply of inoculum having a continuous anaerobic history.

Management of anaerobic shake cultures. Anaerobic shake culture tests were conducted in all-glass equipment in the manner described by Andreasen and Stier ('53).

Lipid stock solution. The fatty acids were stocked as ethanol solutions; for example, 0.705 gm oleic acid per 10 ml ethanol. When 0.1 ml of this solution is used in 25 ml of medium the final concentration is 282 μ g or 1 μ mole oleic acid per milliliter of medium.

The ergosterol stock solution was prepared by combining 0.0625 gm ergosterol and 6.25 ml of surfactant (see below) with sufficient ethanol to make 25 ml of solution. The ergosterol was dissolved by boiling the mixture. The use of 0.1 ml of this solution in 25 ml of medium resulted in a final concentration of 10 μ g or 0.025 μ mole ergosterol per milliliter of medium. The alcohol introduced into the medium by the use of these lipid solutions did not have an adverse effect on the populations obtained. The alcohol concentration was held constant in testing the effect of graded amounts of lipid.

EXPERIMENTAL RESULTS

Experiments employing surfactants as sources of fatty acids. The requirement for unsaturated fatty acid in anaerobic nutrition was first demonstrated when various surfactants were employed for the solubilization of ergosterol.

Those employed were Tween 80 (monooleate ester), Tween 40 (monopalmitate ester) and Tween 20 (monolaurate ester) obtained from the Atlas Powder Company, as well as the non-lipid agent, Triton WR1339 (arylalkyl polyether of phenol) obtained from the Rohm and Haas Company. Commercial grade fatty acids are used in the manufacture of the Tweens. The data obtained are shown in table 1.

TABLE 1

Surfactants as sources of fatty acids. Medium: Basal no. 1. Concentration of surfactants, 7 mg/ml; oleic acid, 4 mg/ml; ergosterol, 70 µg/ml

LIPID IN MEDIUM	POPULATION <i>M</i> cells/ml	LIPID IN MEDIUM	POPULATION <i>M</i> cells/ml
Oleic acid	8	Tween 20	14
Tween 80	4	Tween 20 + ergosterol	48
Tween 80 + ergosterol	200	Tween 20 + Tween 80 + ergosterol	70
Tween 40 + ergosterol	0	Tween 20 + oleic acid + ergosterol	132
Tween 40 + Tween 80 + ergosterol	110	Triton	3
		Triton + ergosterol	25
		Triton + Tween 80 + ergosterol	200
		Triton + oleic acid + ergosterol	200

The following conclusions can be drawn from the data in table 1. (1) Neither the surfactants nor free oleic acid produce significant growth when used separately. (2) When the surfactants are used to solubilize ergosterol, only the Tween 80-ergosterol combination produces good growth. (3) When Tween 20, Tween 40 or Triton are used to solubilize ergosterol, supplementation with Tween 80 or free oleic acid increase the population. These results show that the oleate molecule has a pronounced growth promoting effect under anaerobic conditions, but only if ergosterol is also present. The saturated fatty acids, laurate and palmitate, are virtually inactive and appear to be toxic.

Experiments employing various fatty acids. It was of particular interest to examine various fatty acids for their relative growth promoting activity. Hormel Foundation high-purity acids were used, with the exception of crotonic acid (C.P.) and beta-hydroxybutyric acid (Matheson). All of the acids were employed at a suboptimal concentration of $0.1 \mu\text{mole/ml}$ of medium, while some of them were used at

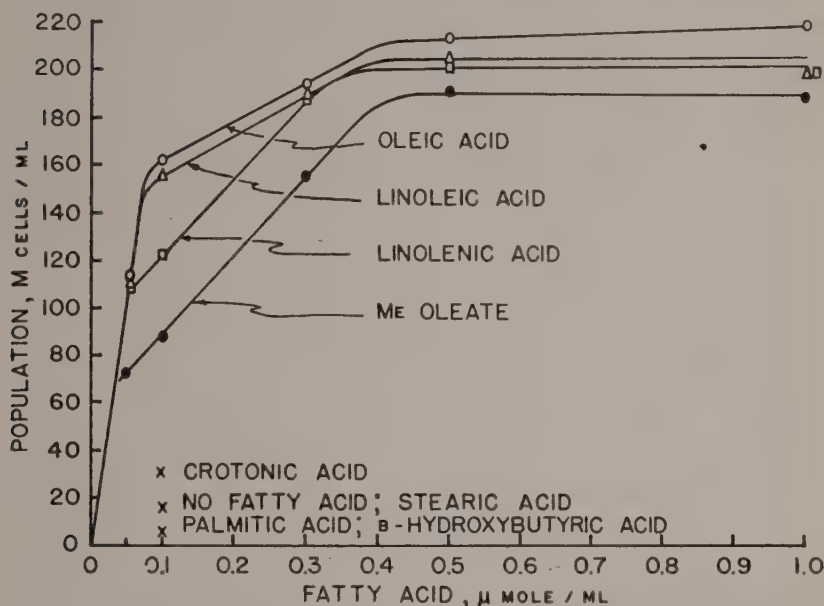


Fig. 1 Activity of various fatty acids. Data were obtained using Basal no. 2 or no. 3 media containing $20 \mu\text{g/ml}$ ergosterol solubilized with Triton, 2 mg/ml . Anaerobic inocula were prepared in similar media.

graded concentrations from 0.05 to $1.0 \mu\text{mole/ml}$. In each of these experiments Triton WR1339 was used to solubilize the ergosterol which was employed in the test media at the supra-optimal concentration of $20 \mu\text{g/ml}$ ($0.05 \mu\text{mole/ml}$). The anaerobic inoculum was grown in a similar medium containing optimal amounts of oleic acid and ergosterol. The populations obtained represent the growth from a single subcultivation in Basal no. 2 or no. 3 media, and are given in figure 1.

The data presented in figure 1 permit several observations. First, it is apparent that only the unsaturated fatty acids are active. Furthermore, the 4 fatty acids that were tested at high concentrations produced stationary populations that are nearly equally great in magnitude. At suboptimal concentrations, however, in the range of 0.05–0.3 μ mole/ml, linolenic acid is less active than oleic and linoleic acids. It appears, therefore, that three double bonds are less effective for growth than one or two double bonds, when the acids are used in the suboptimal concentration range.

It is also apparent from figure 1 that esterification of oleic acid to methyl oleate has an adverse effect on activity in the suboptimal concentration range. It should be mentioned, however, that this adverse effect *may* be due to the presence of Triton. For example, when the ester Tween 80 was used as a source of oleic acid (Andreasen, '53), it was found that all of the esterified acid was available. If Triton was added to the medium, however, all of the acid was not available. As the concentration of Triton was increased, it was necessary to increase the concentration of Tween 80 in order to obtain a given population. This effect of Triton was not observed with free oleic acid. These results indicate that Triton inhibits Tween 80 esterase activity, increasing the requirement for esterified acid, and, therefore, may also increase the requirement for methyl oleate. If this explanation is proved correct, then it must be assumed that the fatty acid is not active in esterified form. This aspect of lipid metabolism needs further investigation.

The C4 unsaturated crotonic acid gives slight growth promotion at a very low concentration (0.1 μ mole/ml), but the population was not improved by using larger amounts. Stearic and palmitic acids are ineffective at the concentrations tested, while palmitic acid is found to be toxic at the higher concentrations (e.g. when used in conjunction with small amounts of oleic acid).

Effect of oleic acid concentration on population. Experiments were performed to determine the optimal concentra-

tion of oleic acid. Basal no. 2 or no. 3 media, containing non-limiting concentrations (0.050 – 0.075 μ mole/ml or 20 – 30 μ g/ml) of ergosterol solubilized with Triton was employed for these experiments. The Triton also solubilized the Hormel Foundation high-purity oleic acid that was added to the medium in varying concentration. It was found that a concentration of 0.2 – 0.4 μ mole oleic acid/ml (56 – 113 μ g/ml) was required to produce maximal stationary populations in the range of 180 – 250 M cells/ml.

Oleic acid was non-toxic over a broad range of concentration. The free acid did not cause a diminution of population when used at concentrations up to 1000 μ g/ml, the maximum tested. Similarly, Tween 80 containing the equivalent of approximately 1750 μ g/ml of oleic acid was not deleterious.

The minimum amount of oleic acid required to produce a given population was determined under conditions where oleic acid was the only medium constituent limiting growth. It was found that an essentially linear relationship exists between population and oleic acid concentration over a population range extending from 0 to *ca* 140 M cells/ml. One μ g/ml of oleic acid produces approximately 6 – 10 M cells/ml in this population range. The latter population represents an oleic acid requirement of *ca* 355 μ moles to produce one million cells, a relatively large amount compared to the ergosterol and vitamin requirements. For example, when determined under similar conditions, the minimum ergosterol requirement is *ca* 84 μ moles/M cells while the requirement for the indispensable vitamin nicotinamide is *ca* 20 μ moles/M cells (Andreasen, '53).

It is worthy of mention that both oleic acid and ergosterol depress the populations obtained in aerated cultures when these lipids are employed at concentrations that are optimal for anaerobic growth.

DISCUSSION

No reports have been found that describe an unsaturated fatty acid requirement for yeast, but there are several in-

stances where fatty acids have been shown to be essential or beneficial to the growth of microorganisms and animals. The reviews of Bloor ('43), Peterson and Peterson ('45), and Knight ('45) contain reports on the essentiality of oleic acid for species of *Lactobacillus*, *Mycobacterium*, *Corynebacterium* and *Clostridium* as well as *Erysipelothrix rhusiopathiae* and *Pityrosporum ovale*. More recent reports have extended this list (cf. Oyama et al., '53). Saturated fatty acids are effective in some cases, however, and the requirement is not always confined to anaerobic conditions. Also, there are instances where the essential fatty acids have been found to be toxic unless some detoxifying agent is added to the medium. Furthermore, the quantitative requirements of oleic acid for optimal growth are so varied that it has been suggested that this lipid may serve different functions. In addition to fatty acid requirements by microorganisms, it is well known that higher forms of life require dietary long-chain unsaturated fatty acids such as linoleic, linolenic or arachidonic acid (Burr, '42). Although apparently all cells contain saturated and unsaturated fatty acids (Bloor, '43; Smedley-MacLean and Thomas, '20) very little is known about the cellular fatty acids other than their essentiality, in some instances, and their utility as a carbon or energy source and their association with various cell structures.

The necessity of unsaturated fatty acids for the growth of certain organisms suggests that these organisms are incapable of forming fatty acids, or of desaturating any saturated acids that may be formed. There is evidence that fatty acid biosynthesis by yeasts and molds is greatly depressed unless ample air is supplied (MacLean and Hoffert, '26; Terroine et al., '26; Halden, '34, and Schmidt, '35). Also, Klein ('51) found that total lipid synthesis in coenzyme A-poor yeast, produced by pantothenate deficiency, is nearly abolished. Klein's observation may be pertinent to anaerobic biosynthesis of lipids if one considers a report by Novelli and Lipmann ('50), who found that yeast must actively oxidize glucose to form useful amounts of coenzyme A from panto-

thenic acid. It is not known, however, whether or not anaerobic yeast contains significant amounts of any fatty acids. Such an analysis is made difficult, under the growth conditions employed in our experiments, by the virtual failure of growth in the absence of added unsaturated fatty acid.

Since the above data shows that the addition of saturated fatty acids to the medium is ineffectual, it is also possible that dysfunction of desaturation mechanisms could be responsible for the failure of anaerobic growth. Recent work summarized by Snell ('51) indicates that biotin and various enzymes are important for the formation of unsaturated fatty acids. Although biotin was found to be indispensable for anaerobic growth, oleic acid could not replace any of the biotin when the biotin was used in suboptimal concentrations (Andreasen, '53). The simultaneous anaerobic requirement for ergosterol may be considered as favoring an assumption that fatty acids are not biosynthesized under anaerobic conditions inasmuch as both sterols and fatty acids are supposedly biosynthesized by similar mechanisms.

We wish to thank the Atlas Powder Company and the Rohm and Haas Company for generous supplies of their surfactants.

SUMMARY

A strain of the yeast *Saccharomyces cerevisiae* requires oleic, linoleic or linolenic acid for anaerobic growth in a chemically defined medium. Linolenic acid is less active than oleic and linoleic acids when the acids are employed at suboptimal concentrations. Ergosterol (or certain other sterols) is also required for anaerobic growth, so that *both* lipids must be added to the defined medium in order for proliferation to occur. It is suggested that the anaerobic requirement for unsaturated fatty acid is due to failure of its biosynthesis under anaerobic conditions.

The defined basal media employed consisted of trace elements, salts, ammonium sulfate, B-vitamins, glucose and

sodium succinate buffer. Ergosterol and the fatty acids were routinely solubilized by the non-lipid surfactant Triton WR-1339.

The amount of oleic acid required to produce stationary populations in the range of 180–250 million cells per milliliter was 0.2–0.4 μ mole/ml. Under conditions where only oleic acid limited growth, the minimum requirement was 1 μ g/ml to produce *ca* 10 M cells/ml, or 355 μ moles/million cells. Oleic acid was not detrimental to anaerobic growth over a broad concentration range, but aerobic growth was depressed at the optimal anaerobic concentrations.

LITERATURE CITED

- ANDREASEN, A. A. 1953 Anaerobic nutrition of *Saccharomyces cerevisiae*. Doctorate thesis, Indiana University.
- ANDREASEN, A. A., AND T. J. B. STIER 1953 Anaerobic nutrition of *S. cerevisiae*. I. Ergosterol requirement for growth in a defined medium. *J. Cell. and Comp. Physiol.*, 41: 23.
- BLOOR, W. R. 1943 Biochemistry of the fatty acids. Reinhold Publ. Corp.
- BROWN, A. J. 1905 The influences regulating the reproductive functions of *S. cerevisiae*. *J. Chem. Soc. (London)*, 87: 1395.
- BROWN, H. T. 1914 Studies on yeast. II. The metabolism of the yeast cell, with special reference to the thermal phenomena of fermentation. *Ann. Bot.*, 28: 197.
- BURR, G. O. 1942 Significance of the essential fatty acids. *Fed. Proc.*, 1: 224.
- HALDEN, W. 1934 *Z. Physiol. Chem.*, 225: 249.
- KLEIN, H. P. 1951 Relation of coenzyme A to steroid and total lipid synthesis in yeast. *Fed. Proc.*, 10: 209.
- KNIGHT, B. C. J. G. 1945 Growth factors in microbiology. *Vit. and Hormones*, 3: 105.
- NOVELLI, G. D., AND F. LIPMANN 1950 The catalytic function of coenzyme A in citric acid synthesis. *J. Biol. Chem.*, 182: 213.
- OYAMA, V. I., H. G. STEINMAN AND H. EAGLE 1953 Nutritional requirements of treponemata V. A detoxified lipide as the essential growth factor supplied by crystallized serum albumin. *J. Bact.*, 65: 609.
- PASTEUR, L. 1879 Studies on Fermentation, Macmillan and Co., London.
- PETERSON, W. H., AND M. S. PETERSON 1945 *Bact. Rev.*, 2: 49.
- SCHMIDT, L. H. 1935 *Am. J. Physiol.*, 111: 138.
- SMEDLEY-MACLEAN, I., AND D. HOFFERT 1926 Carbohydrate and fat metabolism of yeast. III. Nature of intermediate stages. *Biochem. J.*, 20: 343.
- SMEDLEY-MACLEAN, I., AND E. M. THOMAS 1920 The nature of yeast fat. *Biochem. J.*, 14: 483.

- SNELL, E. E. 1951 In *Bacterial Physiology* by C. H. Werkman and P. W. Wilson, Academic Press.
- TERROINE, E. F., AND R. BONNET 1927 *Bull. Soc. Chim. Biol.*, 9: 588.
- WHITE, J., AND D. J. MUNNS 1951 The effect of aeration and other factors on yeast growth and fermentation. *Wallerstein Lab. Comm.*, 14: 199.
- WICKERHAM, L. J. 1951 *Taxonomy of yeasts*. Tech. Bull. no. 1029, U.S.D.A., 56 pp.
- WINDISCH, FRITZ 1932 Die Bedeutung des Sauerstoffs für die Hefe und ihre biochemischen Wirkungen. *Biochem. Z.*, 246: 332.

UTILIZATION OF DISSOLVED CALCIUM BY A PELECYPOD ¹

K. PAMPAPATHI RAO ² AND EDWARD D. GOLDBERG

*Scripps Institution of Oceanography, La Jolla, and the Department of Zoology,
University of California, Los Angeles*

ONE FIGURE

Despite the intense interest exhibited in recent years concerning the nature of uptake and the primary source of calcium for shell formation in marine molluscs, the subject is still controversial. Orton ('25) noticed shell growth in oysters in the apparent absence of food. From similar observations Galtsoff ('34) reported that the amount of calcium assimilated is many times greater than could be stored in the tissues. Fox and Coe ('43) showed that in *Mytilus californianus* the amount of calcium obtained from organic food alone was not sufficient to account for the amount deposited in the shell.

In a comprehensive review of calcification in molluscs Robertson ('41) has stated that the greater part of the shell of marine molluscs is probably absorbed from sea water as calcium and bicarbonate ions. Bevelander and Benzer ('48) have shown that there is a corresponding reduction in the calcium of the growing shell, when the calcium content of sea water is reduced. In a recent study of the process of calcium intake in molluscs, Bevelander ('52) comes to the conclusion that the mollusc can and does utilize calcium in an ionic form in the production of the mineral component of the shell. Since

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²Present address: Andhra University, Waltair, India. Aided by a grant to Dr. Theodore H. Bullock from the National Institutes of Health, U. S. Public Health Service.

these experiments were carried out in unfiltered sea water, they do not necessarily demonstrate the intake of ionic calcium in true solution, for it is known that large amounts of colloidally and otherwise suspended organic and inorganic matter in sea water are ingested by the filter feeders. Further, little or nothing is known about what regions of the organism may be involved in extracting the calcium from the sea water, and about the physical basis of such an intake. The belief that the primary source of calcium is that present in dissolved form is based on indirect evidence and is only inferential. The present investigation indicates that under controlled conditions a source of calcium for the shell-forming marine molluscs is found in true solution in the sea water and that it is taken up in an ionic form by a hitherto unsuspected mechanism.

We wish to express our thanks to Dr. Denis L. Fox, Division of Marine Biochemistry, Scripps Institution of Oceanography, for his encouraging interest in the present study. Our special thanks are due to Dr. Theodore H. Bullock, Department of Zoology, University of California at Los Angeles, for critically reading through the manuscript.

MATERIALS AND METHODS

Young *Mytilus californianus* Conrad, collected from the rocks at La Jolla, were kept overnight in large fingerbowls containing sea water, previously passed through a molecular filter which removed all particles larger than $0.5\ \mu$. These bowls were kept in a constant temperature room ($14^{\circ} \pm 1^{\circ}\text{C}$). Four to 7 mussels, each measuring 15 to 25 mm in length, were transferred to each of 4 fingerbowls, two of which contained filtered sea water, and the other two unfiltered sea water. One milliliter of $\text{Ca}^{45}(\text{HCO}_3)_2$ containing 101 mg of Ca^{++} with an activity of $50\ \mu\text{c}$ was added to the first two and one of the remaining bowls, together containing 1500 ml

of sea water; the last bowl served as a control.³ Since normal sea water contains about 400 mg of calcium per liter, the resulting concentration of calcium in the experimental vessels was about 470 mg/l. When sea water is filtered under suction (as was the case in the present study), carbon dioxide is removed. Such a system will shift the reaction $\text{Ca}(\text{HCO}_3)_2 \rightleftharpoons \text{CaCO}_3 (\text{ppt.}) + \text{H}_2\text{CO}_3$ to the right. To insure such a shift, the pH of the added tracer solution was buffered to around 7.5, and during the entire course of the experiment a stream of air was kept flowing through the water in the aquaria.

The mussels were allowed to remain in the aquaria for periods of three to 36 hours. Whole animals were sectioned and radioautographs were made by the inverted autograph technique (Belanger, '50). Similar experiments were performed wherein one of the filtered sea water aquaria contained suspended colloidal graphite (Aquadag). In another case only $\text{Ca}^{45}\text{CO}_3$ was added and was kept in suspension by the aeration. In these experiments small areas were scraped away from the shell margin without injuring the soft tissues in order to induce accelerated calcium assimilation for the consequent regeneration.

Cleaned inert shell-valves as well as pieces of soft tissue were kept in experimental aquaria to serve as control references for estimating the likely exchange between active calcium of the medium and the inactive calcium of the shell or tissues.

In studying the role of mucus in other intertidal forms, freshly collected specimens of the tunicates, *Ciona intestinalis*, *Styela montereyensis*, the mussel *Mytilus edulis*, and the anemone *Anthopleura xanthogrammica* were maintained overnight in filtered sea water and then transferred to finger-bowls containing freshly filtered sea water containing the radioactive tracer. After a couple of hours, mucus collected

³ The Ca^{45} used in this investigation was supplied by the Oak Ridge National Laboratories on allocation from the Isotopes Division, U. S. Atomic Energy Commission.

from each animal was washed in fresh tracer-free sea water and then in distilled water. One-half milliliter of mucus was transferred to a copper planchet and dried under an infra-red lamp for the assay of the radioactivity. For control purposes, mucus obtained from freshly collected members of all the above named species was washed in clean sea water and then placed for 15 minutes in filtered sea water containing the tracer. After this interval, the mucus was taken out and treated similarly for assaying of the activity.

At regular intervals during the course of an experiment, individual mussels were removed from the experimental aquaria. Regions of the body were dissected away, thoroughly washed in freshly filtered sea water (without added tracer) and mounted on planchets to be dried for counting. Special attention was paid to the gills, mantle margin and transverse sections of various regions of the body. Whole animals were also mounted and dried.

Materials for sectioning were fixed in Bouin's fluid or in 10% formaldehyde, cut in paraffin and stained with basic fuchsin or Masson's trichrome stain.

RESULTS

Mytilus californianus, kept in filtered sea water with radioactive calcium ion shows a measurable intake of this cation from the medium into the tissues other than the gills within two hours, as shown by measuring the radioactivity of various parts of the body and by radioautographs of sectioned material. Gills of these animals, on the other hand, show activity within one-half to one hour in the experimental sea water. They remain the most active fraction of the body at any time thereafter. Mantle margin showed relatively high activity compared with the other parts of the mantle, but activity was highest near the regenerating region. Radioautography of sectioned material revealed extremely high concentrations of calcium in the regenerating areas of the mantle margin and these can be very sharply localized to the region of injury (fig. 1). Freehand sections (about 0.5 mm

thick) of mussels three to 6 hours after the start of the experiment, passing through the body at the level of the heart, showed high activity compared to those passing through more anterior and more posterior parts of the body. The observed

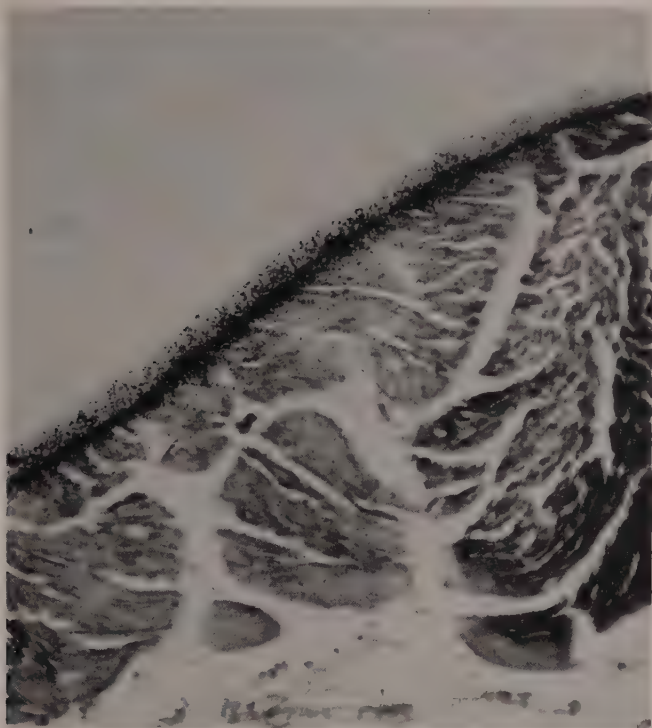


Fig. 1 Autoradiograph of the longitudinal section of the mantle margin just behind the posterior adductor in a region of repair of injury to the shell.

accumulation of active calcium in the tissues of the experimental mussels was very much greater than the total activity exhibited by dead shell and pieces of soft parts used as controls.

No detectable increase in the intake of calcium was observed in the experiments using graphite, or suspended calcium carbonate or unfiltered sea water. Thus freehand sections about 0.5 to 1 mm in thickness of such mussels after 24 hours

in experimental aquaria gave 449 ± 35 counts/min., while those of mussels from filtered sea water (without any particulate material in it) gave 512 ± 37 counts/min. A slight increase in the number of counts may be noticed in the latter case. This may be due to the variables involved in such a procedure, such as the thickness of the sections and the normal variability of the counter and the disposition of the material under the end-window.

Of great interest was the fact that mucus, either *in situ* or removed from the animal, took up great concentrations of ionic calcium from solution, probably by adsorption (table 1).

TABLE 1
Uptake of radioactive calcium by mucus
(See Materials and Methods for technic)

ANIMAL	WEIGHT OF IONIC CALCIUM TAKEN UP BY MUCUS	
	<i>In situ</i> , exposed for several hours	Removed from body exposed for 15 mins.
	mg. Ca/ml wet wt. ¹	mg. Ca/ml wet wt. ¹
<i>Ciona intestinalis</i>	0.278 ± 0.020	0.256 ± 0.017
<i>Styela montereyensis</i>	0.430 ± 0.034	0.406 ± 0.031
<i>Mytilus edulis</i>	0.370 ± 0.025	0.352 ± 0.022

¹ Converted from activity; 5000 counts per min. represent 0.234 mg Ca⁴⁵.

It is significant that the mucus from different species (including non-shell forming ones) collected from about the same intertidal zone reacted in a similar manner inasmuch as it took up large amounts of ionic calcium from solution.

DISCUSSION

These experiments demonstrate that an extensive source of calcium for *Mytilus californianus* (and probably other shell-bearing marine lamellibranchs) may be found in true solution in the sea water. The amount of calcium that the animals could obtain is considerable as can be seen from the following. In these experiments about 5000 counts/min. represent 0.234 mg of calcium. Body slices of about 1 mm thickness gave

about 512 counts/min., thus containing about 0.024 mg of calcium. These slices represent about one-seventh to one-tenth of the soft parts of the 15–20 mm mussels used. That means each mussel has accumulated from the medium at least 0.17 to 0.24 mg of calcium in 24 hours. According to Fox and Coe ('43) mussels of such length contain 0.156 to 0.211 mg of calcium in their tissues. Consequently, the present investigation demonstrates that calcium in true solution is taken in by *Mytilus californianus* in highly significant quantities, equivalent to more than the total content of the tissues in 24 hours and possibly much sooner in agreement with Wilbur and Jodrey ('52).

The initial transfer of calcium from the sea water to the mussel occurs at the mucus surface of the gill, perhaps by a process of adsorption, where the adsorbing surface is continuously in an unsaturated state with respect to the medium, and it is presumably maintained in such a state by a constant renewal of the surface of adsorption. In such a system the number of adsorbed ions at any given time is determined by the number present in the environment. In his review Robertson ('41) concludes that "secretion of shell material goes on almost automatically provided sufficient calcium is available" (of course, other conditions like temperature being within normal limits). If this be so, perhaps herein lies an explanation for the variability in shell weight encountered under different circumstances, and in different localities in the same species (Rao, '53).

The extraordinary capacity of the mucus surface in several intertidal invertebrates to adsorb ionic calcium is of interest. It has been shown by Goldberg, McBlair and Taylor ('51) that the mucus sheet of tunicates adsorbs vanadium from aqueous solution. Perhaps several ionic species are adsorbed from the medium onto the mucus surface in many marine invertebrate species. Since there are many naturally occurring glycoproteins (mucins), there are probably specific enzymes in the digestive tracts of various invertebrates capable of disintegrating the mucus and extracting only the required

adsorbed components and incorporating them into their systems, the rest being thrown out. Or, in forms like the oyster, the results of Jodrey ('53) suggest that direct transfer of calcium from the mucus to the shell across the mantle margin is possible. But the exact nature of the mechanism is still unknown.

According to Fox and Coe ('43) the mussel adds shell only when in "an actively feeding condition"; if poor nutrition exists, the supply of calcium cannot be taken advantage of. Even so the present experiments show that, not only is *Mytilus* able selectively to extract calcium in solution from the sea water, but is also capable of directive localization in extremely well defined areas of the mantle where an active regeneration has been induced by scraping off tiny bits of shell-margin. This need not mean that there is any perceptible net growth taking place under our laboratory conditions. The high concentrations of calcium observed in these sites contrasts with the low concentrations reported by Jodrey ('53) in normal mantle edge.

The present results can supplement in these respects the studies of Wilbur and Jodrey ('52) and Jodrey ('53). In addition, they indicate that the 97.6% of mantle calcium which Jodrey calculates to be inactive does in fact turn over sometime within 24 hours.

SUMMARY

It has been shown that an extensive and important source of calcium for *Mytilus californianus*, and possibly other marine lamellibranchs, is dissolved in the sea water. The mucus sheet of the gills is shown to provide an adsorbing surface, which is in a constant state of renewal. Mucus of various other marine invertebrates has also been shown to behave in a similar way. Specific enzyme systems in the gut of the particular organism are believed to aid in the extraction of specific constituents adsorbed to the mucus, depending on the needs of the organism.

It has been shown that the mussel is capable of concentrating great amounts of calcium in the areas of active deposition, as in repair of local injury to the shell.

LITERATURE CITED

- BELANGER, L. F. 1950 A method for routine detection of radio-phosphates and other radioactive compounds in tissues. The inverted autograph. *Anat. Rec.*, 107: 149-159.
- BEVELANDER, G. 1952 Calcification in molluscs. III. Intake and deposition of Ca^{45} and P^{32} in relation to shell formation. *Biol. Bull.*, 102: 9-15.
- BEVELANDER, G., AND P. BENZER 1948 Calcification in marine molluscs. *Biol. Bull.*, 94: 176-183.
- FOX, D. L., AND W. R. COE 1943 Biology of the California sea mussel (*Mytilus californianus*). *J. Exp. Zool.*, 93: 205-249.
- GALTSOFF, P. S. 1934 The biochemistry of the invertebrates of the sea. *Ecol. Monogr.*, 4: 481-490.
- GOLDBERG, E. D., W. McBLAIR AND K. M. TAYLOR 1951 The uptake of vanadium by tunicates. *Biol. Bull.*, 101: 84-94.
- JODREY, L. H. 1953 Studies on shell formation. III. Measurement of calcium deposition in shell and calcium turnover in mantle tissue using the mantle-shell preparation and Ca^{45} . *Biol. Bull.*, 104: 398-407.
- ORTON, J. H. 1925 The conditions for calcareous metabolism in oysters and other marine animals. *Nature*, 116: 13.
- RAO, K. P. 1953 Rate of water propulsion in *Mytilus californianus* as a function of latitude. *Biol. Bull.*, 104: 171-181.
- ROBERTSON, J. D. 1941 The function and metabolism of calcium in the Invertebrata. *Biol. Rev.*, 16: 106-133.
- WILBUR, K. M., AND L. H. JODREY 1952 Studies on shell formation. I. Measurement of the rate of shell formation using Ca^{45} . *Biol. Bull.*, 103: 269-276.

ADDENDUM

Since the above account has gone to press, an interesting paper on strontium uptake in some molluscs and polychaetes has been published (Fretter, '53), in which some points are of interest in this connection. It was shown that in the nudibranch *Acanthodoris pilosa*, there is a relatively rapid accumulation of strontium (and presumably of calcium also) from the surrounding water. It is likely that the mucus covering over the body concentrates the ions from the medium which then pass through the general surface of the body, while "relatively few" ions are found in the lumen of the gut. In *Mytilus edulis* it was shown that strontium made a direct entry to the ciliated epithelium of the gills, although such an entry of ions into the body was not considered to be the major or primary route. It was concluded

that the gut is the more important area for ingress of ions into the tissues, the ions having been directed there by the feeding currents. We have shown above that mucus is the agent by means of which ions in solution are collected from the medium and transported to the gut, this being the more important area for ingress of ions into the tissues. Entry of ions directly into the mantle tissue does occur (Hirata and Jodrey, '53), though, presumably, to a lesser degree.

FRETTER, V. 1953 Experiments with radioactive strontium (^{90}Sr) on certain molluscs and polychaetes. J. Mar. Biol. Assn. U.K., 32: 367-386.

THE DEPENDENCE OF UNIT DISCHARGES ON FREQUENCY AND INTENSITY OF STIMU- LATION OF THE MOTOR CORTEX IN THE MACAQUE¹

J. HYDE, C. M. RIGGLE AND E. GELLHORN
*Laboratory of Neurophysiology, Physiology Department,
University of Minnesota, Minneapolis*

FIVE FIGURES

In a series of investigations Gellhorn and collaborators (1945-1951) have studied the gross responses of striated muscles to stimulation of the motor cortex in the macaque. In this and the following paper an attempt is made to analyze some of the factors which determine the behavior of individual motor neurons under these conditions and to correlate unit discharges with the gross response of the muscle.

METHODS

The gross electromyogram (EMG) was recorded from the belly of the muscle by means of two copper wires sewn into the muscle as in previous investigations of this series. For the recording of unit potentials two pairs of bipolar needle electrodes or Gesell's monopolar "floating" electrodes were inserted close to the tendon of the anterior tibialis or the biceps brachialis muscle. The superficial fascia being removed, the electrode tips were placed just beneath the surface. The selection of a place suitable for recording unit potentials was aided by listening to the discharges on a type 210 Offner audio unit and observation on a Dumont cathode-ray oscilloscope. The recording was photographic, a double beam cath-

¹ These studies were aided by a contract between the Office of Naval Research, Department of the Navy, and the University of Minnesota.

ode ray oscilloscope being used making it possible to obtain records from two pairs of electrodes simultaneously. The influence of the signal from one channel on the output of the other was found to be negligible. The low frequency cut-off of the 140-A amplifier was set at .002 to prevent gross fluctuation of the base line due to movement; the high cut-off was at zero (i.e., no additional cut-off was imposed on the amplifier). Recording was at a film speed of 10 cm/sec., a time signal indicating intervals of 0.1 sec. Ten macaques anesthetized with Dial-urethane were used. The motor cortex was stimulated with condenser discharges or, in a few cases, square waves pulses. For further details see earlier papers from this laboratory.

RESULTS

I. The dependence of unit discharges on intensity and frequency of stimulation of the motor area

The records obtained under these conditions were studied with respect to their frequency and amplitude. Moreover, since particularly with increasing degrees of excitation of the motor cortex more units appeared, the mutual relation of the discharges of several units was determined.

If a site of the motor cortex was stimulated with a low voltage and/or with a low frequency a minimal response occurred in the gross electromyogram (EMG), whose amplitude seems to be related to the number of activated units (Loofbourrow, '48). Unit discharges appearing under these circumstances were of equal magnitude and showed a gradual increase in the rate of discharge during the 5-sec. period of stimulation. The greatest unit activity coincided with the maximal amplitude of the EMG and this phase of activity is referred to as the *optimal* period of discharge. The frequency of stimulation varied between 11.4 and 102 per second and the intensity between one and 6 volts. The rate of discharge of individual units showed considerable variations

in these experiments. In the majority the maximal rate² of discharge was low and little altered by an increase in the frequency of stimulation. Thus in one experiment the maximal rate varied between 20 and 24/sec. as the frequency of stimulation was increased from 33 to 102/sec. (the voltage being kept at 3.5 volts). In another experiment unit activity was recorded while the motor cortex was stimulated at 33 and 83/sec. with intensities ranging from 3.0 to 5.7 volts. The maximal rate of discharge varied between 30 and 33/sec. in the series with stimulation at 33/sec. and between 27.4 and 30.0 in that involving stimulation at 83/sec. However, occasionally the maximal rate of discharge attained the frequency of stimulation when the latter was not higher than 30 to 50/sec. In a few instances even higher maximal rates have been observed. They occurred mostly in the form of two or three discharges at the stimulus intervals. Other units discharged simultaneously at a lower rate. This fact as well as the observation that the maximal rates of discharge occurred only during a small fraction of the "optimal period" accounts for the lack of a direct relation between the maximal rate of unit discharges and the amplitude of the EMG.

These results are amplified in table 1. Although a moderate increase in the frequency of discharge of the fastest unit is noted in some experiments (see especially those involving the relatively high voltages at frequencies of 11.4 and 102/sec.), the maximal rate is not a determining factor of the total response of the muscle. Thus at 5.7 volts with a frequency of stimulation of 11.4/sec. the maximal rate of discharge of the fastest unit is 28/sec., and this same rate occurs on stimulation with 2.6 volts at 83/sec.; but the total response and also the number of discharging units is much greater in the latter condition. Moreover, in the experiments with frequencies of 33/sec. and 53/sec. the maximal rate in each series seems to be constant although with increasing voltage

² The maximal rate is determined by the reciprocal value of the shortest interval found between two discharges of a single unit. The frequency is defined by the number of spikes of a single unit per second. The latter may vary without a significant change in the former.

the number of discharging units and the gross response as indicated by the amplitude of the EMG increase. This indicates clearly that not the maximal firing rate but the number of discharging units accounts for the increase in gross response (and muscle tension) which occurs under conditions

TABLE 1

The influence of increasing intensity and frequency of stimulation on the number of the responding motor units and their frequency of discharge¹

FREQUENCY VOLTS	NUMBER OF DISCHARGING UNITS				
	11.4	33	53	83	102
2.1	..	1	0	0	0
2.6	..	2	..	5	..
3.0	0	2	3	4	5
3.5	..	5	..	6	7
4.2	0	5	5	5	7
4.9	2	6	4	..	8
5.7	3	..	..	..	8

Maximal rate of any unit in c/s

FREQUENCY VOLTS	11.4	33	53	83	102
2.1	..	..	0	0	0
2.6	..	30	..	28	..
3.0	0	32	48	28	36
3.5	..	33	..	44	51
4.2	0	32	53	35	51
4.9	12	34	53	..	51
5.7	28	..	..	..	54

Gross response in mm

FREQUENCY VOLTS	11.4	33	53	83	102
2.1	..	..	3	0	0
2.6	..	7	..	17	..
3.0	4	8	8	11	9
3.5	..	10	..	18	10
4.2	5	9	10	25	10
4.9	7	12	12	..	11
5.7	8	..	..	..	13

¹ The experiment was performed on a Macaque in Dial anesthesia. A biceps point of area 4 was stimulated with condenser discharges of 7.7 ms. duration.

of increasing excitation of the motor cortex. Table 1 furnishes examples that either increasing the frequency of stimulation at constant voltage or increasing the voltage while the frequency of stimulation is kept constant causes recruitment of additional motor units.

If the maximal rate of discharge of an individual unit is relatively insignificant, it is to be expected that the total number of spikes during the optimal period would be a good indicator of the central excitation since it depends on the number of the activated units and their average frequency of discharge. This inference is supported by the experiments illustrated in table 2. The activity of the units was recorded during two series in which the voltage was increased from 2.1 to 5.7 while the frequency of stimulation was 33 and 83/sec. Although there were marked changes in the gross EMG response the discharge frequency of the individual units was not significantly altered. However, the total number of unit discharges showed a good correlation with the gross response.

It is concluded that under conditions of stimulation of the motor cortex with increasing intensity and/or frequency the total number of discharges is correlated with the increased gross response of the muscle. This number depends primarily on the number of activated neurons. Another important, although obvious, factor is the duration of the discharge. With increasing central excitation the rate of recruitment is accelerated and, in addition, the duration of the discharge continues for a longer time beyond the period of stimulation.

In addition it should be said that the higher threshold units which were activated as the degree of central excitation and the gross EMG response increased were often distinctly taller than the spikes which appeared on lesser degrees of stimulation. This is interesting inasmuch as Kugelberg and Skoglund ('46) have made similar observations under conditions of voluntary innervation and stimulation of motor nerves in man.

II. Frequency specificity in the motor cortex and its reflection in the discharge of single units

It was shown by Hyde and Gellhorn ('51) that the reactivity of various sites of the motor cortex to a series of stimuli varying in frequency shows fundamental differences as judged by the degree of contraction of the muscles acti-

TABLE 2

The influence of increasing intensity and frequency of stimulation on the total unit discharges, individual units, and the gross response (EMG) of the biceps muscle¹

Total number of unit discharges in 1.1 sec.

VOLTAGE FREQUENCY	2.1	2.6	3.0	3.5	4.2	4.9	5.7
33		27	28	65		58	
83	31	68	80	71	68		69

Number of unit A discharges in 1.1 sec.

VOLTAGE FREQUENCY	2.1	2.6	3.0	3.5	4.2	4.9	5.7
33		15	15	11		11	
83	15	16	17	17	15		20

Number of unit B discharges in 1.1 sec.

VOLTAGE FREQUENCY	2.1	2.6	3.0	3.5	4.2	4.9	5.7
33		0	0	22		19	
83	0	19	26	20	20		18

Number of unit C discharges in 1.1 sec.

VOLTAGE FREQUENCY	2.1	2.6	3.0	3.5	4.2	4.9	5.7
33		0	0	22		19	
83	0	19	26	20	20		18

Gross EMG response in mm

VOLTAGE FREQUENCY	2.1	2.6	3.0	3.5	4.2	4.9	5.7
33		5	6	8		9	
83	2	11	14	15	14		16

¹ Macaque in Dial anesthesia. Stimulation of a biceps focus with condenser discharges (pulse duration 7.7 ms.).

vated. Different cortical foci which on stimulation elicit a contraction in the same muscle may have widely different optima of frequency of stimulation. Furthermore, two or more muscles brought into contraction by stimulation of a single cortical focus may also show different optimal frequencies for the relative maximal shortening or tension. This work was repeated and expanded by recording single unit responses together with the gross response.

The unit response to stimulation of several cortical sites with different optimal stimulus frequencies for elicitation of a maximal gross EMG from tibialis was investigated in several animals. The results in each case were consistent. The same units responded to stimulation of cortical sites in the leg area separated by as much as 8 mm (the greatest distance tried). If a maximal gross tibialis response was elicited by a medium frequency (e.g., 40–50/sec.) of stimulation, this same frequency evoked the maximal unit response,³ both in number of responding units and in frequency per unit, these factors diminishing at higher or lower stimulus frequencies. Further the response would continue throughout a longer portion of the *applied* stimulation at the “optimal” frequency, and the tendency to an afterdischarge was greater.

A different cortical site with a frequency optimum of e.g., 80–100/sec. would elicit from the same muscle an increased unit response as well as gross EMG as the stimulus frequency was increased to this level. These results are summarized graphically in figure 1 while a sample of such records is shown in figure 2. The maximal rate at which the individual units discharged in these experiments was independent of stimulus frequency. Since the number of spikes in a constant

³ The characteristics described by the term “maximal unit response” are (1) largest number of units firing; (2) highest frequency of discharge in the individual units (where this varied appreciably in response to different stimulus frequencies); and (3) greatest number of spikes in a constant proportion of the period of stimulation. The last number is determined by the number of the discharging units and the number of discharges of each unit. The number of discharges is not necessarily related to the maximal rate of firing since the latter may last only for a very short period of time.

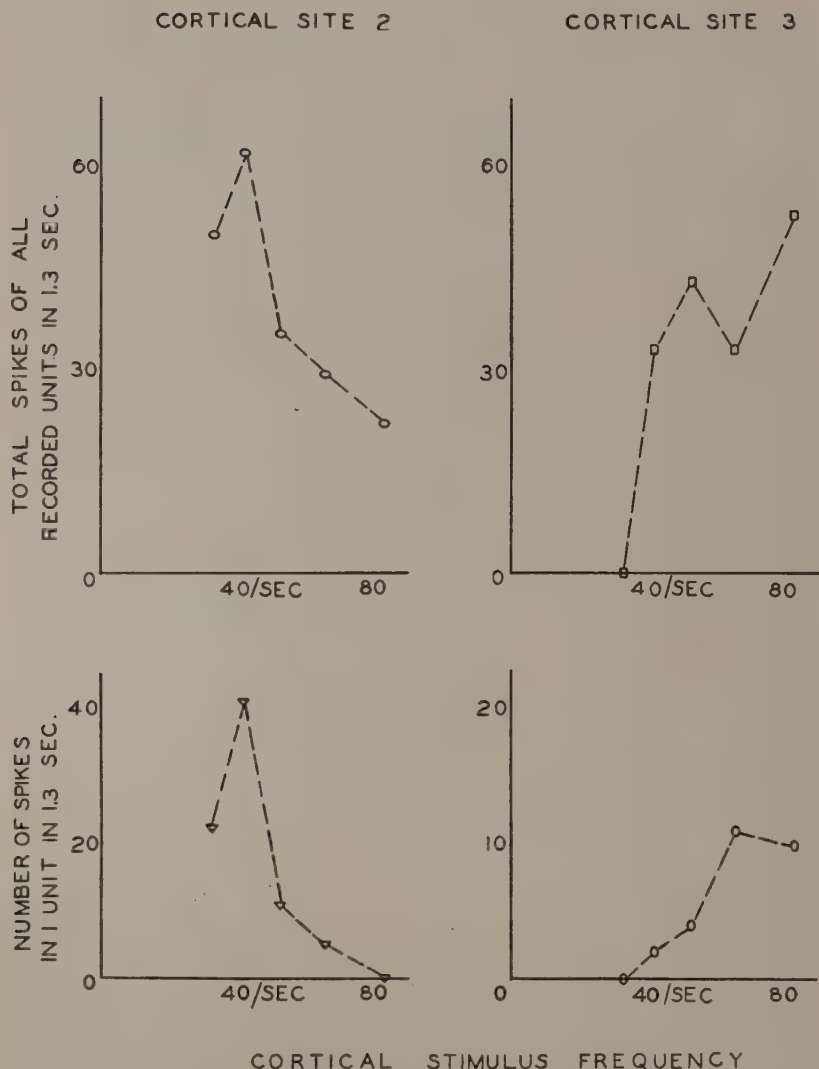


Fig. 1 Graphical representation of unit responses to stimulation of two cortical sites at varied frequencies. Oscillographic responses in figure 2. Upper graphs show number of spikes in all recorded units recorded in the 1.3 sec. of maximal response to stimulation of the two cortical sites, as function of cortical stimulus frequency. Lower graphs show the number of spikes of one unit (the largest of figure 2) in the same period as a function of stimulus frequency. Cortical site 2 elicited maximal response in tibialis units when stimulated at about 42/sec.; site 3 (3 mm lateral to site 2) showed a larger response with higher stimulus frequencies.

time interval showed a relation to the stimulus frequency and to the gross response of the muscle, this was the factor chosen for the graphical representation of different optimal stimulus frequencies at different cortical sites in figure 1. In the first



Fig. 2 Single unit responses to stimulation of two cortical sites with varied frequencies in the macaque. A-E, stimulation at 1.7 volts (for 5 seconds, pulse duration 7.7 milliseconds) of a cortical site 3 mm lateral to midline, 3 mm anterior to central sulcus. F-J stimulation at 2.1 volts (otherwise as above) at a cortical site 3 mm lateral to site 2.

pair of graphs the total number of spikes of both large and small units in 1.3 sec. of the stimulation period is plotted as a function of stimulus frequency. The number of spikes of one particular unit is similarly plotted in this figure. It is

quite obvious that the response of the same unit elicited from two different cortical sites may show fundamentally different frequency characteristics. At cortical site 2 the optimal response is at 42/sec. and at site 3 at 83/sec. of stimulation.

Another experiment from a different macaque is graphically illustrated in figure 3. One cortical site elicited a greater response with low stimulus frequencies, the response of the units (like the gross tibialis EMG) dropping off as the stimulus frequency was raised; the second site showed a maximal effect with a higher frequency of stimulation, the optimum varying in the different units between 66 and 83/sec., and the number of the spikes falling off a 102/sec. Examination of the films illustrates the tendency for the units to discharge at the stimulus rhythm or at the higher frequencies of stimulation at sub-fractions of the stimulus rate, resulting in an apparent "pick-up" of the stimulus frequency in the response.

The first pair of graphs in figure 3 shows the total number of spikes recorded by one pair of electrodes during three seconds of the response, plotted as a function of stimulus frequency for the two cortical sites. In the second pair of graphs one particular unit is similarly graphed. It can be seen that the maximal response as indicated by number of spikes is at a lower stimulus frequency for cortical site 3 than for site 4, a result paralleling the gross tibialis EMG's. The maximal rate of this same unit is plotted in the third set of graphs as a function of stimulus frequency. The maximal rate, as shown in this set of graphs, is apparently not a reliable indicator of magnitude of response.

III. Pulse duration and excitability of the motor cortex

Since only a few data are available on the relation of pulse duration to the responsiveness of the motor cortex, some experiments are presented in this section which are concerned with the relation of the pulse duration to unit discharges as well as to the gross response (EMG) of the

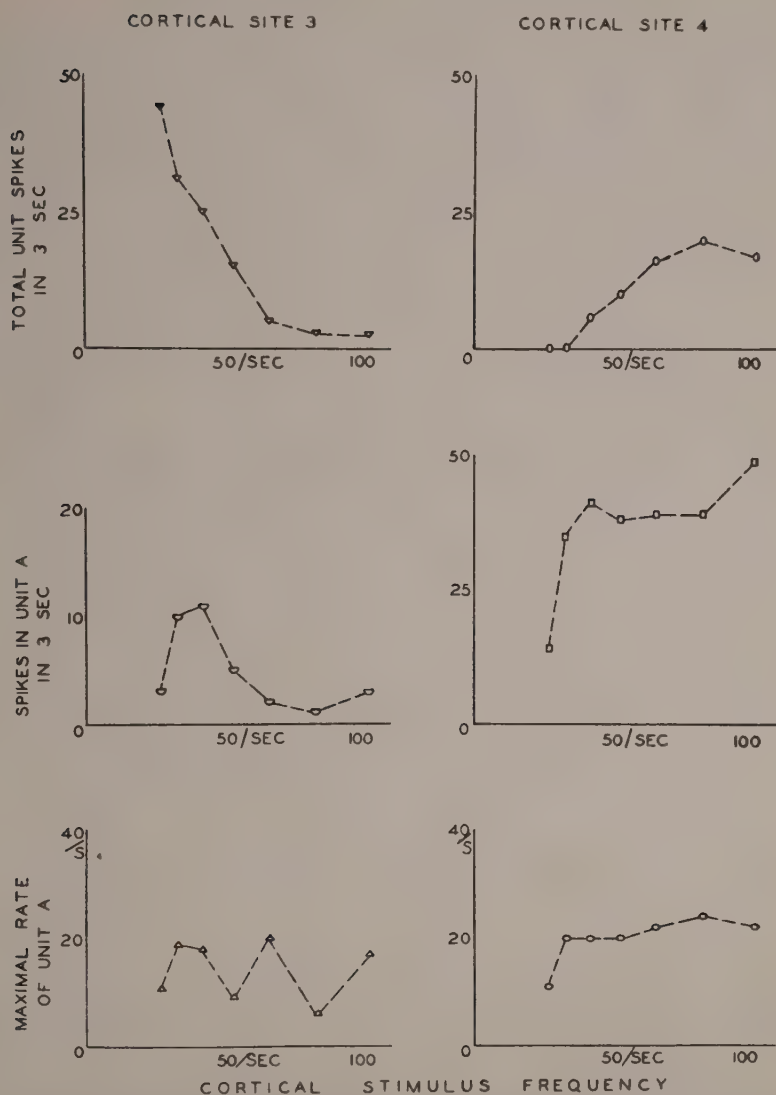


Fig. 3 Effect on motor unit responses of stimulation of two cortical sites with varied frequencies at 3 volts (site 3) and 3.5 volts (site 4). Macaque; oscillographic records are omitted. Upper pair of graphs represent number of spikes of all the units in one channel (i.e., recorded with one pair of electrodes) in the 3 sec. of maximal activity during the 5 sec. cortical stimulation, as function of cortical stimulus frequency at the two sites. Middle graphs show a similar plotting for a single unit. In the lower graphs the maximal rate of this unit is graphed as a function of cortical stimulus frequency.

muscle. On three monkeys the motor cortex was stimulated at different frequencies and varying pulse durations, and the effect on unit discharges was determined. A typical experiment is presented in figure 4 showing that the maximal

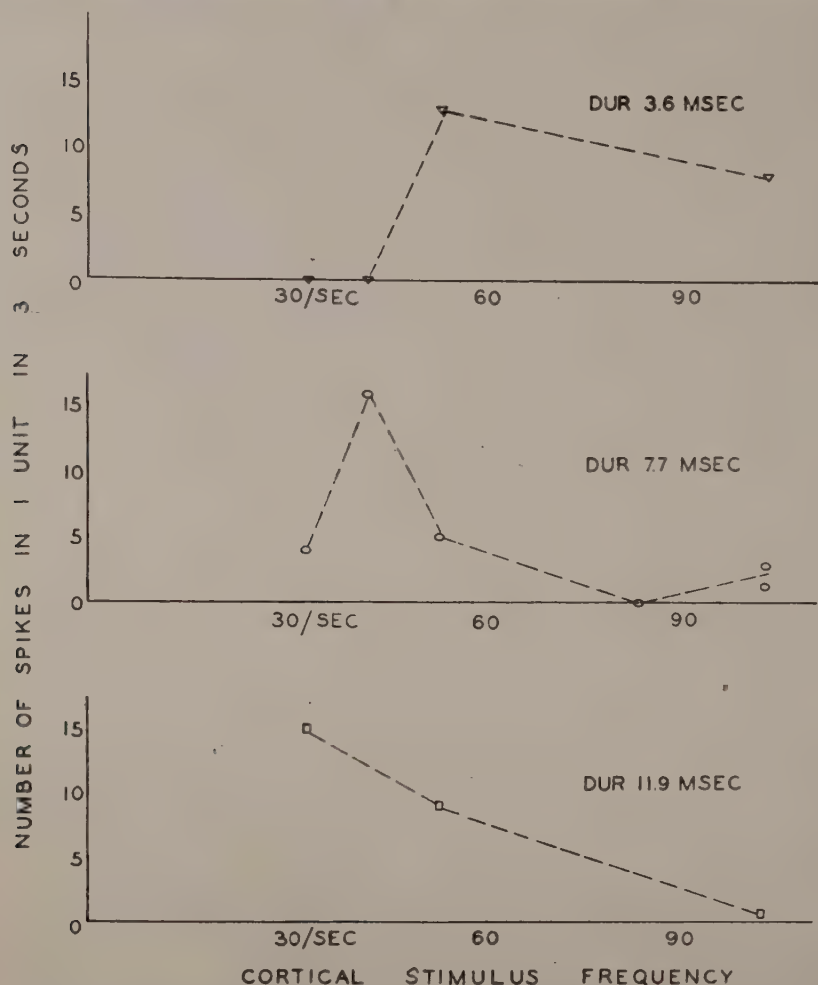


Fig. 4 Graphical representation of effect of pulse duration on response of a motor unit to cortical stimulation at different frequencies in the macaque. Number of spikes of a single unit during the 3 sec. of maximal activity in response to a 5-sec. stimulation of a site in area 4, plotted as a function of frequency of stimulation for three different pulse durations. Maximal response is at higher frequency with a shorter pulse duration.

number of spikes discharged by a single unit in three seconds remains practically constant in the three sets of tests. However, this maximum shifts from a higher to a lower frequency of stimulation as the duration of the condenser discharges is increased. The results of (two) experiments, which have in common that the optimal response at a short pulse duration occurs at a relatively high frequency, are shown in table 3. It seems to follow from these experiments that the optimal frequency of stimulation and the pulse duration are interrelated.

TABLE 3

Summary of effect of pulse duration on optimal cortical stimulus frequency

MONKEY	UNIT	PULSE DURATION	OPTIMAL FREQUENCY
		<i>ms</i>	<i>per second</i>
1	A	2.0	42
		3.6	33
		7.7	33
		11.9	27
	B	2.0	42
		3.6	33
		7.7	27
		11.9	27
2	All	3.6	53
		7.7	42
		11.9	33

DISCUSSION

If suprathreshold stimuli are applied to the motor cortex for several seconds, there is a gradual increase in the frequency of discharge of single units recorded from striated muscles, and also an increase in the number of discharging units. The total number of unit discharges recorded per unit of time during the optimal period of contraction of the muscle (indicated by the largest amplitude of the EMG) increases with increasing excitation of the motor cortex regardless whether this increase is due to an augmentation of the voltage or an alteration in frequency or

pulse duration. The study of unit discharges under these conditions reveals further that individual units recorded from the same microelectrode may show wide variations in frequency of response. This phenomenon is particularly marked on stimulation of the cortex with higher intensity and frequency since under these conditions in addition to the units which discharge at a low frequency some units may appear which, at least for brief periods, show a rate of activity equal to that of the stimulus frequency. However, it should be stressed that in general the frequency of unit discharges is low, varying between 20 and 30/sec. in spite of relatively wide variations in intensity and frequency of stimulation. It is worthy to mention that Landau ('52) on stimulation of the pyramidal tracts with widely different frequencies at threshold intensity obtained unit discharges in the muscle of a low frequency.

In the majority of the experiments the discharge was asynchronous as far as it can be judged from the records showing up to three or 4 units. In some records, however, and particularly during marked responses (high amplitude of the electromyogram) there was a tendency to a synchronous discharge. The various units appeared at constant intervals after the stimulus artifact although an individual unit may discharge only after every second or third stimulus. These observations occurred in animals which were apparently in a similar condition as far as depth of anesthesia and intensity of stimulation are concerned as those showing asynchronous discharges. They suggest that with increasing degree of central excitation the neuronal discharge may become increasingly synchronous. It is interesting to note that this tendency to synchrony was observed by Adrian and Bronk ('28) in discharges of the phrenic nerve under conditions of forcible respiration.

It should be pointed out that on near threshold excitation spikes of low amplitude are recorded and that with increasing intensity of stimulation spikes of higher amplitude appear. Kugelberg and Skoglund ('46) have made similar observa-

tions on electrical stimulation of a peripheral nerve in man and in voluntary contraction of a muscle. These authors refer also to earlier recordings of voluntary activity in single units (Smith, Denny-Brown and Pennybacker) and to different possible interpretations which might be made of this finding that low amplitude spikes appear at a lower threshold of activation than larger spikes. This phenomenon awaits further experimental clarification; meantime it is noted that the same relationship among units of different size obtains under conditions of motor cortical stimulation, voluntary activation, and peripheral nerve stimulation.

It has been shown by Hyde and Gellhorn ('51) that cortical neurons of the motor area show certain frequency characteristics. The frequency optimum of stimulation was found to be different for different muscles activated from the same cortical site and also the response of a single muscle may show different optimal frequencies when it is activated from different cortical sites. The latter observation has been confirmed through the recording of unit potentials. Figures 2-4 illustrate the fact that the same unit may show different frequency optima when it is activated from two different cortical sites of area 4. This indicates clearly that the frequency selectivity of motor responses elicited by cortical stimulation is not a characteristic of the motor horn cells but rather must lie "upstream" from them.

That the optimal cortical stimulus frequency is not a resonance (or purely frequency) phenomenon is indicated by the experiments on relation of pulse duration to frequency. When a longer pulse duration is used, the optimal frequency for a particular cortical site is lower than with a shorter pulse duration.

SUMMARY

Oscillographic recordings of single motor unit responses (of tibialis anterior and biceps brachi muscles) to cortical stimulation of area 4 with altered stimulus parameters were obtained in the macaque. Analysis of the records leads to the following conclusions:

1. The frequency of discharge of a single motor unit is in general independent of the cortical stimulus frequency and often confined to a range of 20–30/sec.

2. Increasing the intensity or frequency of stimulation leads to recruitment of new units. Consequently the total number of spikes discharged in one second increases. It is this number and not the maximal rate of discharge of an individual unit which parallels the amplitude of the gross response (electromyogram) and thereby the muscle tension.

3. There are wide variations in the frequency of discharge of individual units recorded from the same microelectrode under conditions of stimulation of the motor area. These individual differences contribute to the asynchrony of the contraction. With greater central excitation units of increasing amplitude appear. There is also under these conditions a tendency to synchronous discharges of several units which occur at a constant interval after the stimulus artifact although an individual unit may discharge only after every second or third stimulus.

4. The “optimal” frequency of stimulation may not be the same at two different cortical sites for the same motor units; a unit which shows the greatest frequency and most prolonged discharge to cortical stimulation of moderate (40–50/sec.) frequency at one site may show this optimal response to higher (50–100/sec.) or to lower (25–35/sec.) frequencies of stimulation of another cortical site.

5. This “optimal” frequency of stimulation is dependent on the duration of the condenser discharge. Other conditions being equal the optimal frequency decreases with increasing duration of the impulse.

APPENDIX

In further series of experiments on two monkeys the relation of pulse duration to the gross response of the muscle was investigated. The motor cortex was stimulated over a frequency range from 10 to 200 per second while the pulse

duration was varied between 1 and 25 milliseconds rectangular discharges being used.

In these experiments a shift in the frequency optimum did not occur probably due to the fact that the foci stimulated gave a frequency response with the optimum at 20/sec. and that the response of the muscle to the next lower frequency

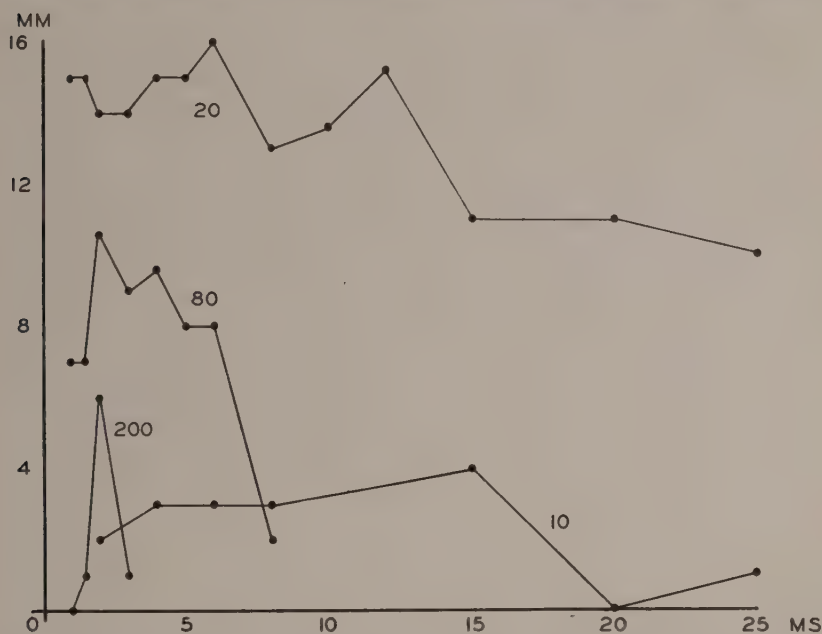


Fig. 5 Effect of increased pulse duration on the gross amplitude of EMG at various frequencies of stimulation of the motor cortex in a macaque. The EMG was recorded from the extensor carpi radialis (1 millivolt = 5 mm). Stimulus frequencies per second indicated on the graphs. Ordinate: amplitude of the EMG in millimeters; abscissa: pulse duration in milliseconds.

(10/sec.) was very slight. However, it was noted that the effectiveness of cortical stimulation increased with increasing pulse duration and that the optimum became increasingly broader with decreasing frequency of stimulation (fig. 5). Moreover, the experiments show that a change in frequency is a more potent means of increasing the effectiveness of stimulation of the motor cortex than is a change in pulse duration.

LITERATURE CITED

- ADRIAN, E. D., AND D. W. BONK 1928 The discharge of impulses in motor nerve fibers. Part I. Impulses in single fibers of the phrenic nerve. *J. Physiol.*, *66*: 81-101.
- BOSMA, J. F., AND E. GELLHORN 1947 The organization of the motor cortex of the monkey based on electromyographic studies. *Brain*, *70*: 127-144.
- DENNY-BROWN, D., AND J. B. PENNYBACKER 1938 Fibrillation and fasciculation in voluntary muscle. *Brain*, *61*: 311-334.
- GELLHORN, E. 1948 The influence of alterations in posture of the limbs on cortically induced movements. *Brain*, *71*: 26-33.
- 1949 Proprioception and the motor cortex. *Brain*, *72*: 36-62.
- 1950 The validity of the concept of multiplicity of representation in the motor cortex under conditions of threshold stimulation. *Brain*, *73*: 267-274.
- GELLHORN, E., AND D. A. JOHNSON 1950 Further studies on the role of proprioception in cortically induced movements of the foreleg in the monkey. *Brain*, *73*: 513-531.
- HYDE, J., AND E. GELLHORN 1951 The physiological effect of variations in the stimulus frequency on the motor cortex of the monkey. *Brain*, *74*: 432-442.
- KUGELBERG, E., AND C. R. SKOGLUND 1946 Natural and artificial activation of motor units—a comparison. *J. Neurophysiol.*, *9*: 399-412.
- LANDAU, W. M. 1952 Patterns of movement elicited by medullary pyramidal stimulation in the cat. *EEG Clin. Neurophysiol.*, *4*: 527-536.
- LOOFBOURROW, G. N. 1948 Electrographic evaluation of mechanical response in mammalian skeletal muscle in different conditions. *J. Neurophysiol.*, *11*: 153-168.
- MURPHY, J. P., AND E. GELLHORN 1945 Multiplicity of representation versus punctate localization in the motor cortex: an experimental investigation. *Arch. Neurol. Psychiat.*, *54*: 256-273.
- SMITH, O. C. 1934 Action potentials from single motor units in voluntary contraction. *Am. J. Physiol.*, *108*: 629-638.

THE EXCITATION OF SINGLE STRIATED MUSCLE FIBERS¹

JULIUS PRAGLIN²

Department of Physiology, University of Illinois, Urbana

ELEVEN FIGURES

Keith Lucas ('06, '07) first reported that the strength-duration curve (SDC) of frog striated muscle, taken with large electrodes, consisted of three discontinuous segments — the β excitability with an excitation time or chronaxie of 50 to 100 μ sec., the γ excitability with a chronaxie of 0.1 to 1.0 msec. and the α excitability with a chronaxie of about 10 msec. Lucas concluded that the β excitability arose from stimulation of the end-plate, the γ excitability from nerve stimulation and the α excitability from direct stimulation of the muscle fibers.

Rushton ('30) however, could not confirm the existence of Lucas' β excitability in whole muscle, while Kuffler ('45) did not succeed in demonstrating this excitability in single sartorius nerve-muscle fiber preparations.

Contemporaneously, Hill ('10, '35a,b), Lapicque ('07), Blair ('41), Rashevsky ('38) and Monnier ('34) had developed excitation theories which, if it is assumed that accommodation is negligible, give in common, a theoretical SDC of the form

$$V/R = 1/(1 - A \exp^{-t/B}), \quad (I)$$

where

V = the voltage necessary to excite at duration t .

R = the rheobase voltage,

A and B are constants.

¹ A preliminary report of this work appeared in *Fed. Proc.*, vol. 11, no. 1 (1952).

² Present address: Laboratory for Research in Ophthalmology, Lakeside Hospital, Western Reserve University, Cleveland, Ohio.

In Lapicque's, Rashevsky's, Monnier's and Hill's ('35a) theories of excitation A does not appear and may be considered equal to *one*. In Hill's ('10) theory and in Blair's theory A may take any value. B equals Hill's k , Monnier's T_1 , while the reciprocal of B equals Blair's and Rashevsky's k .

In testing the experimental validity of equation (I), Hill ('35b) recommends plotting the experimental data on logarithmic ordinates and comparing the experimental curve obtained with a theoretical curve calculated from equation (I) with A equal to *one*. Blair, on the other hand, rearranges equation (I) to obtain

$$\log_e V/(V - R) = kt + C \quad (\text{II})$$

where

$$k = 1/B$$

$$C = -\log_e A.$$

Experimentally, $\log_e V/(V - R)$ is plotted against t . If the theory holds, the result is a straight line with slope k and intercept C .

Blair ('41) has presented evidence from various sources that C must have a value other than *zero* (A have a value other than *one*) in most cases for experimental results to agree with equation (II). Jinnaka and Azuma ('22) and Ramsey and Street ('38) found that the SDC of single muscle fibers agreed with equation (II) with C not equal to *zero*. Lapicque ('31) has pointed out that Rushton's α -curves for whole muscle fit the simple exponential relationship ($A=1$) only rarely. There is, however, no proof that whole muscle α -curves can be fitted by any form of equation (I).

Hill ('35b), nevertheless, has shown for frog nerve, that equation (I), with A equal to *one*, fits the facts so well that A may be omitted from the equation. The results of Rushton on frog nerve analyzed by Blair ('41), support Hill's findings. Prosser and Chambers ('38) found satisfactory agreement between equation (I) with A equal to *one* and experimental results obtained with the giant squid axon. Le Fevre ('50) also found equation (I) to apply, in the form of A equal to *one* to

squid nerve, although better agreement was found if accommodation was also considered.

This paper will attempt to decide whether the SDC of single muscle fibers can be predicted by equation (I) and further, if this is so, whether or not A need be different from *one* in magnitude. The question of whether Lucas' β excitability exists will also be considered.

MATERIALS AND METHODS

The experimental materials were normal, denervated and curarized sartorii and normal and curarized semitendinosi of *R. pipiens*, *R. catesbiana*, and *R. clamitans*. Unless exception is noted, the muscles were equilibrated in cold Ringer's solution³ for at least 12 hours from the time of dissection to the time of the experiment. Curarization, where employed, was accomplished by the injection of 0.1 to 0.2 cm³ of Tubocurarine (Abbot) (3 mg/cm³) into the dorsal lymph sac and by equilibrating as above, but in curare-Ringer's solution (10⁻⁵ gm Tubocurarine per cubic centimeter). Denervated sartorius muscles were used two weeks after excision of the motor nerve.

Four experimental methods were used:

1. Semitendinosus or sartorius muscles, suspended in a layer of paraffin oil, were stimulated by surface electrodes of bare platinum wire, 0.13 mm in diameter and 4.0 mm apart. Recording of the action potential of the most excitable fiber at the muscle surface was accomplished by bipolar, platinum recording electrodes each 25 μ in diameter. The shock artifact was reduced by a grounded platinum hook between the stimulating and recording electrodes. No evidence of polarization could be found with electrodes larger than about 100 μ in diameter.

2. In other experiments Ag-AgCl-wick electrodes, used for stimulation, confirmed the fact that the bare platinum electrodes did not introduce any polarization error, although the

³ NaCl, 6.5 gm; KCl, 0.14 gm; CaCl₂, 0.12 gm; NaHCO₃, 0.2 gm; NaH₂PO₄, 0.01 gm; triple distilled, pyrogen-free H₂O, 1.0 l; pH adjusted to 7.4.

α excitation constant was increased due to the larger tip diameter of these electrodes (300 to 500 μ).

3. Single muscle fibers were isolated from the semitendinosus muscle and stimulated in Ringer's solution by means of large Ag-AgCl-gelatin electrodes placed a few millimeters from each end of the fiber. Undamaged fibers gave all-or-none mechanical responses and consistently repeatable observations during the experiment.

4. Whole muscles were suspended in oil as in method (1) but the stimulating electrodes were placed on the motor nerve at a distance of about 1 cm from the muscle.

Strength-duration curves were taken on all preparations with square-wave stimuli supplied by a multivibrator-type pulse generator over a duration range of 0.03 to 3000 msec. The rise time of the stimulating pulse was approximately 5 μ sec. with no overshoot. Where electrical recording of the action potential was required as the end-point of stimulation (methods 1, 3, 4), the artifact was minimized by means of a radio frequency carrier transformer similar to the type described by Dubbert and Schmitt ('49). This device effectively isolated the stimulating and recording circuits while preserving the D. C. component of the stimulus. For direct stimulation through solution where the mechanical response of the fiber was observed as the end-point (method 2), coupling of the stimulus to the solution was by means of a cathode-follower-connected 6L6 beam tetrode. Due to the low resistance of the Ringer's solution, the current requirements did not permit durations of less than 0.1 msec. to be used when stimulating through solution.

The direct measurement of accommodation using method (1) was accomplished by superimposing brief testing shocks on a square-wave conditioning pulse at various times after the onset of the conditioning pulse. The same wire electrodes were used for the conditioning and testing pulses which were produced by two separate stimulators and coupled into the common stimulating circuit by means of two R.F. transformers with their secondaries connected in parallel. The interval be-

tween the start of the conditioning pulse and the entry of the testing pulse was timed by a unistable multivibrator. The action potential, initiated by the testing shock, was the criterion for stimulation. Utilization times were calculated from cathode-ray photographs of the action potentials.

At the end of each experiment several points, including the rheobase, were repeated at random. If any of these repeated determinations differed from the original determinations by more than 5%, the entire experiment was discarded.

All SDC's presented in this paper are interpreted by Hill's method of double logarithmic ordinates. An exponential curve, as would be obtained from equation (I) with A equal to one, is fitted to the experimental points plotted with the voltage necessary to excite as the ordinate and the stimulus duration as the abscissa. Where a break in the experimental points occurs, each branch of the curve is fitted to the theoretical curve. The excitation constant B , from equation (I) is determined from the position of the curve relative to the time axis. Accommodation data are presented with logarithmic time abscissa and a linear "testing pulse voltage" ordinate. There was no attempt to test the fit of these accommodation data to any theoretical equation, although, on the assumption that the late phase of accommodation follows an exponential course, Hill's λ and Rashevsky's m were approximated from the experimental data.

EXPERIMENTAL RESULTS

(1) *Motor nerve stimulation.* The strength-duration curves of the motor nerves of the semitendinosus and sartorius were obtained by method (4) above. The SDC of a single nerve fiber could be recorded since the method permitted discrimination of the response of a single motor unit. Figure 1 A shows one of four experiments performed with the semitendinosus and figure 1 B shows one of two experiments performed with the sartorius. The average B -values were 0.23 (range 0.15 to 0.29) and 0.15 msec. (0.14 and 0.15 msec.) respectively. These values are in agreement with the classical results of Lucas and

Rushton for the whole muscle γ excitability. The fit to an exponential curve is excellent and thus the results also agree with Hill's ('35) findings with whole frog nerve.

(2) *Stimulation of curarized muscles with small electrodes.* In the following experiments the SDC's of single fibers were recorded by method (1) above. Figures 3 A and 3 B illustrate

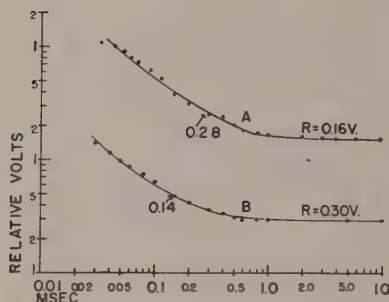


Figure 1

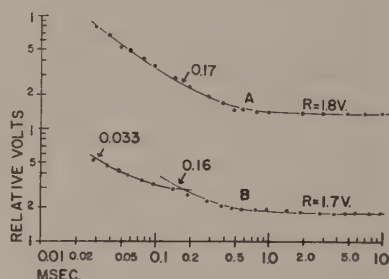


Figure 2

Fig. 1 Strength-duration curves of motor nerves. In part *A* the motor nerve of the semitendinosus is stimulated with wire electrodes. In part *B* the motor nerve of the sartorius is stimulated with wire electrodes. (*A*, experiment of Oct. 20A; *B*, experiment of Oct. 26B.) The voltage ordinate is relative but can be converted into absolute units by reference to the rheobase voltage (*R*) indicated above each curve. The rheobase value given is that found at the beginning of a given experiment. The arrows with the accompanying numerals refer to the *B*-value of each curve segment in milliseconds. The abscissa is the stimulus duration in milliseconds.

Fig. 2 Strength-duration curves of normal muscles. In part *A*, the semitendinosus muscle is stimulated at the pelvic end with wire electrodes. In part *B*, the sartorius muscle is stimulated at the pelvic end with wire electrodes. (*A*, experiment of Oct. 27C; *B*, experiment of Nov. 11.) The method of plotting is the same as in figure 1.

two of seven experiments on the semitendinosus and figures 4 A and 4 B illustrate two of three experiments on the sartorius with small wire electrodes which show clearly a marked deviation from an exponential SDC at short durations.

In the above experiments, the muscles were equilibrated in curare-Ringer's solution overnight before use; however, the possibility that the curare had lost effect during the equilibration period and a discontinuity due to nerve stimulation had

not been eliminated was precluded by two experiments performed on freshly curarized semitendinosi, which gave the same results as muscles equilibrated overnight. Figure 3 A illustrates one of these experiments.

Wick electrode experiments did not differ qualitatively from wire electrode experiments except for the fact that the excitation constant of the portion of the SDC occurring at longer durations was increased. One wick electrode experiment (method 2) is illustrated in figure 3 C.

Not all experiments performed as described above yielded a two segment SDC. Figures 4 C and 4 D show two of eight experiments with the curarized semitendinosus and figures 5 A and 5 C show two of three experiments with the curarized sartorius in which only a smooth, exponential SDC was found. This variation in results was not methodological, but, as shown in table 1, was correlated with the season during which experiments were performed.

It can be seen that the number of experiments showing discontinuities drops off rapidly during the winter months.

It can be seen from figures 1 through 5 that the exponential function is closely followed at durations in excess of 0.15 msec. In cases where a smooth SDC is found with curarized muscle, the α excitation constant is taken as the time constant of the exponential curve which is used to fit the experimental points.

TABLE 1

Per cent discontinuities observed in curarized sartorius and semitendinosus muscles (combined results) as a function of season. Small electrode experiments only

MONTH	NUMBER OF SDC EXPERIMENTS	PER CENT EXPERIMENTS SHOWING DISCONTINUITIES
September 1951	5	100
October	7	85
November	6	50
December	none	..
January 1952	9	11
May	2	100

This time constant has been defined as B in equation (I). In cases where the SDC shows a discontinuity, both portions of the curve are fitted to the exponential curve as in figures 2 B, 3, 4 A and 4 B. The portion of the curve occurring at earlier times is termed, after Lucas, the β excitability, while the portion of the curve occurring at longer times is taken to be the same α excitability as is found in experiments displaying no discontinuities. A Fisher "t" test applied between the α excitation constants of 8 semitendinosus experiments showing no

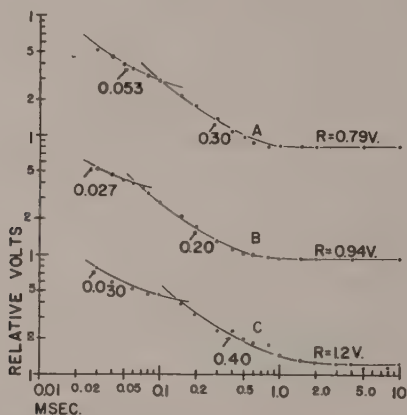


Fig. 3 Strength-duration curves of curarized semitendinosus muscles. In parts A and B stimulating electrodes are platinum wires. In part C the electrodes are Ag-AgCl-wick electrodes. (A, experiment of Oct. 23; B, experiment of Oct. 7; C, experiment of Sept. 25B.) The method of plotting is the same as in figure 1.

discontinuity and 9 experiments showing a discontinuity revealed no significant difference between α excitation constants in the two cases. The average α excitation constant for the semitendinosus with small electrodes was found to be 0.17 msec. with a range of 0.09 to 0.3 msec. The average β B-value was 0.032 msec. with a range of 0.015 to 0.054 msec. Wick electrodes caused no significant change in the β excitability constant but, in common with the results of Watts ('24) and Grundfest ('32), caused an increase in the α excitation constant (average 0.57 msec., range 0.40 to 0.70 msec.) due to the increased electrode area.

The average α B -value for the sartorius was for 5 experiments 0.20 msec. with a range of 0.13 to 0.30 msec. The average β B -value was 0.034 msec. for three experiments with a range of 0.028 to 0.040 msec. The statistical separateness of the α and β classifications for both the semitendinosus and sartorius is affirmed by a "t" test p-value of less than 0.01.

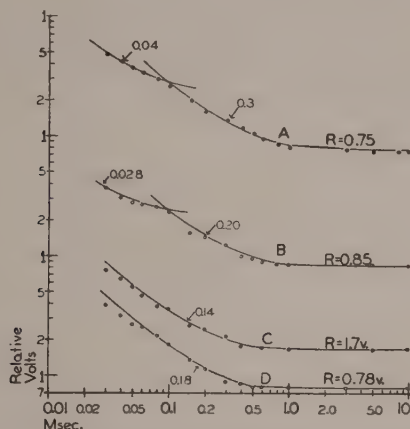


Fig. 4 Strength-duration curves of curarized muscle. In parts *A* and *B*, the sartorius and in parts *C* and *D* the semitendinosus is stimulated with small wire electrodes. (Part *A*, experiment of Nov. 25; *B*, experiment of Nov. 26A; *C*, experiment of Jan. 29; *D*, experiment of Jan 26A.) Method of plotting is the same as in figure 1.

(3) *Stimulation of uncurarized muscle with small electrodes.* Figure 2 A shows one of four experiments performed on normal semitendinosus muscles as described under *method one*. Although the experiments were performed during a time when at least 90% of experiments on curarized muscle showed discontinuities, no discontinuities were ever found with uncurarized semitendinosus muscles. This fact is interpreted to mean that stimulation (pelvic end) of uncurarized semitendinosus muscles is equivalent to motor nerve stimulation. Sartorius pelvic-end stimulation (fig. 2 B) in two experiments yielded results identical to discontinuous curves found with curarized sartorius muscles. The classical finding of Lucas that pelvic-

end stimulation of the sartorius muscle yields only non-nervous stimulation is therefore supported by these data.

(4) *Denervated sartorius muscles.* Figures 5 B and 5 D show two of four experiments performed on denervated sartorius muscles stimulated with wire electrodes. All 4 experiments showed only α excitabilities not significantly different from the α excitabilities of normal and curarized sartorius muscles. These experiments only demonstrate that denervation does not affect the α excitation constant. However, the

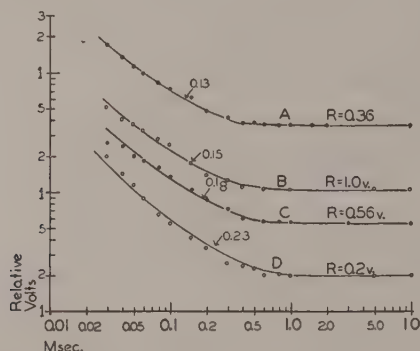


Fig. 5 Strength-duration curves of curarized and denervated sartorius muscles stimulated with wire electrodes. In parts *A* and *C* are illustrated results with curarized sartorius muscles and in parts *B* and *D* are illustrated results with denervated sartorius muscles. (*A*, experiment of Nov. 12; *B*, experiment of Jan. 20; *C*, experiment of Nov. 16; *D*, experiment of Jan. 22.) The method of plotting is the same as in figure 1.

lack of a discontinuity in these experiments is not significant since these experiments were done during the month of January when only 10% of experiments performed showed discontinuities.

(5) *Utilization times.* The utilization time of curarized semi-tendinosus muscles was found to average 2.2 msec. with a range of 1.9 to 2.4 msec. for three determinations. Pelvic-end stimulated sartorius muscles gave values of 2.2 and 2.7 msec. for two determinations. Utilization times were measured with just threshold stimulation.

(6) *Direct measurement of accommodation.* In figures 6 to 8, the testing-pulse voltage necessary to excite is plotted as ordinate and the interval between the onset of the conditioning pulse and the introduction of the testing pulse is plotted as abscissa. In all cases the conditioning pulse was of a duration longer than the conditioning-testing-pulse interval. The magnitude of the conditioning pulse (as indicated in the figure in

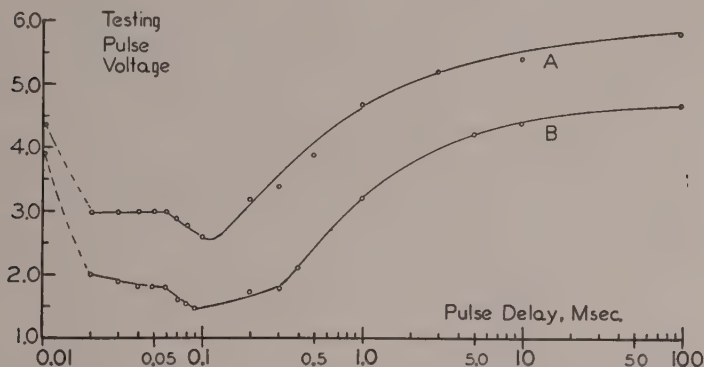


Fig. 6 Accommodation studies of curarized frog sartorius muscles stimulated with small electrodes. (*A*, experiment of Jan. 30C, rheobase = 0.84V., conditioning pulse = 0.75V. *B*, experiment of Jan. 30A, rheobase = 1.0V., conditioning pulse = 0.88V.) The interval between the onset of the conditioning pulse and the introduction of the testing pulse is plotted as abscissa while the ordinate is the testing pulse voltage necessary to excite. The first point in each plot is the voltage necessary to excite in the absence of a conditioning pulse. A dotted line joins the first point to the others, since, properly, the first point is at zero time after the beginning of the conditioning pulse and cannot be properly represented on a logarithmic scale.

question) was between 80 and 90% of the experimental rheobase. The first point on each plot is the value of the testing shock necessary to excite with no applied conditioning pulse. Figure 7 illustrates three of four experiments performed with curarized semitendinosus muscles while figure 6 illustrates two of three experiments performed on curarized sartorius muscles.

The results are qualitatively the same in all cases. There is an initial plateau, which, however, is significantly lower in

voltage than the voltage necessary to excite in the absence of the conditioning pulse. At about 60 to 80 μ sec., there is a sharp discontinuity in the curve due to a sudden decrease in the threshold at that point. As the elapsed time between the onset of the conditioning pulse and the introduction of the testing pulse is further increased, the threshold rises gradually to a value somewhat above the value needed to excite with no conditioning pulse.

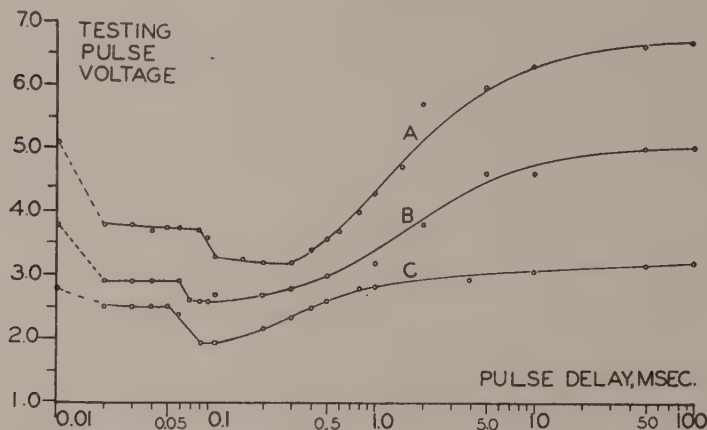


Fig. 7 Accommodation studies of curarized semitendinosus muscles. In parts *A* and *C* wire electrodes were used, in part *B*, wick electrodes were used. (*A*, experiment of Oct. 15, rheobase = 0.56V., conditioning pulse = 0.50V.; *B*, experiment of Jan. 9, rheobase = 0.29V., conditioning pulse = 0.25V.; *C*, experiment of Jan. 7, rheobase = 0.41V., conditioning pulse = 0.36V.) The method of plotting is the same as in figure 6.

Figures 8 A and 8 B show sartorius muscles which have been denervated for two weeks. The initial plateau which was present in experiments with curarized muscles is absent in these denervated preparations. Three experiments of this type were performed.

Hill's accommodation constant, λ , estimated from these curves would be about 1 msec. and no greater than 4 msec. for both the semitendinosus and the sartorius.

(7) *Strength-duration curves of isolated single muscle fibers of the semitendinosus.* Figure 9 illustrates the results of two of four experiments on normal, isolated muscle fibers stimulated through solution with large Ag-AgCl gelatin electrodes [method (3) above]. The discontinuity found in these SDC's, taken with large electrodes, was abolished by curare in all cases studied. Figure 10 presents two of five experiments in which curare abolished the discontinuity in the SDC. These results are in agreement with Rushton's classical results with

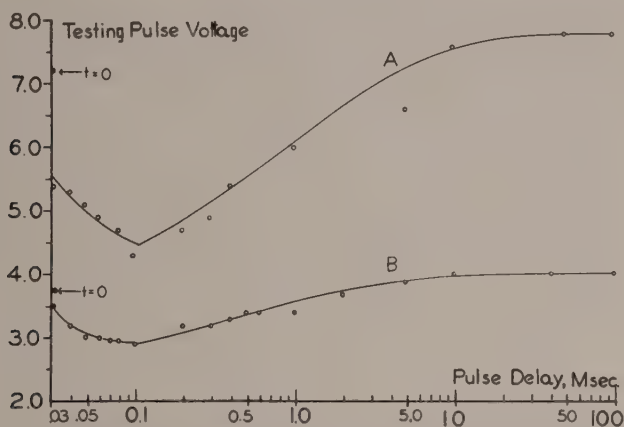


Fig. 8 Accommodation studies of two-week denervated sartorius muscles stimulated with wire electrodes. (A, experiment of Jan. 28, rheobase = 0.75V., conditioning pulse = 0.67V. B, experiment of Feb. 1, rheobase = 1.1V., conditioning pulse = 0.90V.) The point $t = 0$ marks the voltage necessary to excite with no conditioning pulse. Otherwise, the method of plotting is the same as in figure 6.

whole muscle; however, the present data further affirm that, unlike most whole muscle SDC's, both portions of the single muscle fiber SDC obey the exponential equation very well. It may be further pointed out that the average B -value for the γ excitability of these isolated fibers (0.27 msec., range 0.13 to 0.30 msec.) is not significantly different (p -value between 0.8 and 0.9) from the excitation constant of the semitendinosus motor nerve. The average α excitation constant for uncurarized muscle stimulated with large electrodes was 7.5 msec. (range 5.0 to 10.1 msec.). For curarized single fibers the aver-

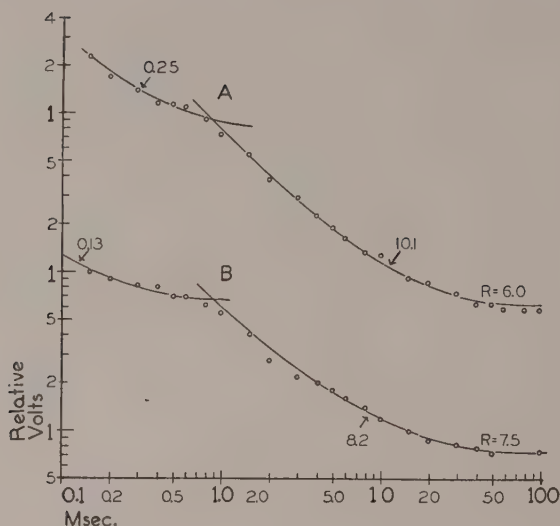


Fig. 9 Strength-duration curves of single, isolated, semitendinosus fibers stimulated in solution with large electrodes. (*A*, experiment of Dec. 4; *B*, experiment of Dec. 14.) The method of plotting is the same as in figure 1.

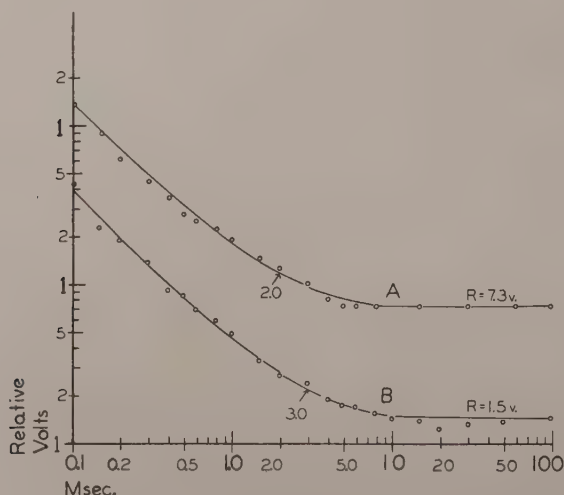


Fig. 10 Strength-duration curves of single, isolated, curarized semitendinosus fibers stimulated in solution with large electrodes. (*A*, experiment of Dec. 17; *B*, experiment of Nov. 28.) The method of plotting is the same as in figure 1.

age B -value was 1.8 msec. (range 1.0 to 3.0 msec.). The present data therefore also show a significant change in the α excitation constant with curare.

DISCUSSION AND CONCLUSIONS

The experimental data presented here support the view that a simple, exponential curve with one constant is sufficient to describe the single-fiber α -SDC. Nevertheless, more careful consideration has been given to Blair's ('41) view-point that two constants must be used to describe the SDC of single muscle fibers. The experiments listed above on single isolated fibers and on fibers stimulated with microelectrodes, were also analyzed by Blair's method but with the additional procedure that the value of $\log_e V/(V - R)$ was calculated twice for each value of t using the upper and lower limits of rheobase (R) value found during a given experiment. In figure 11, the data of figure 5 A on curarized muscle is replotted as an example of the above-outlined procedure. Line A is fitted to the open circles and line B to the solid circles by the method of least squares. Line A was calculated using the smaller and line B the larger rheobase value as indicated in the figure caption. It can be seen that the intercept is of doubtful significance. All data of this paper and the data of Ramsey and Street ('38) on single isolated muscle fibers similarly yielded intercepts or values of Blair's C which were of doubtful significance.

It has been found that, with small wire electrodes, curarized muscle displays a departure from the exponential curve in about 50% of the experiments at short durations. This variation in results has, however, been correlated with season. It is likely, nevertheless, that the portion of the SDC previous to the discontinuity in experiments with wire electrodes is identical to Lucas' β excitability. The β excitation constant of about 0.03 msec. reported here is close to the value of about 0.05 msec. reported by Lucas for the chronaxie of his β excitability. The value of the α chronaxie for capillary electrodes has been reported by Grundfest ('32) for single fibers of the retrolingual

membrane to be about 0.12 msec. Kuffler ('45) obtained α chronaxies of between 0.1 and 0.2 msec. for single sartorius fibers stimulated with small electrodes. The average B -value for all experiments performed in this work with small electrodes corresponds to an α chronaxie (using Hill's conversion factor of $0.67B = RC$) of 0.11 msec. and hence is in very good agreement with the values here reported.

If the departure from the exponential curve at short durations is to be interpreted as the β excitability, the method used

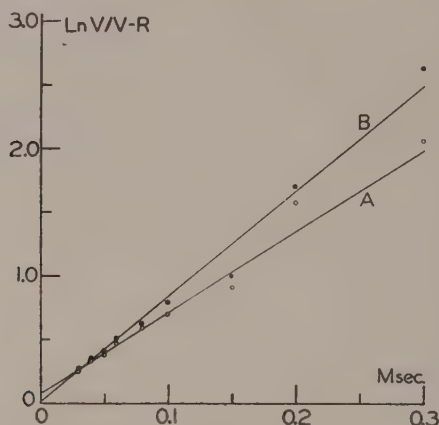


Fig. 11 The data of figure 5 A are replotted by Blair's method. Line A is fitted to the open circles and line B to the closed circles by the method of least squares. In A the rheobase equals 0.36V., in B the rheobase equals 0.38V. The explanation of the figure is in the text.

by Kuffler ('45) to detect the β excitability, that of empirically drawing a smooth curve through the experimental points was invalid; for, unless the fit to an exponential curve was used as a criterion, the β excitability often would not be noticed (see fig. 2 B, for example).

While the classical picture of the variation of the α excitability with electrode size has been well supported by the data presented here, it has been shown above that the β excitability does not vary in excitation constant despite a 300% increase in the corresponding α excitation constant. Unfortunately tech-

nical difficulties did not permit the use of durations short enough to record the β excitability with isolated single fibers. However, it may be pointed out that Lucas, using large fluid electrodes, recorded β excitabilities with whole muscle of the same order of magnitude as reported here although the reported α excitability of Lucas' experiments was 10 to 100 times greater than the α excitation constant found here with small electrodes.

The accommodation studies presented in figures 6 and 7 on the sartorius and semitendinosus, when interpreted in the light of Hodgkin's ('38), Katz' ('37, '48), Hodgkin and Rushton's ('46), and Cole and Curtis' ('41) results on the local response in various tissues, furnish the needed proof that the β excitability is a separate excitatory event. From the records of these workers it is seen that the events of excitation consist first of a charging of the membrane capacity to a critical level at which time the local response begins to rise from the catelectrotonic crest, growing in proportion to the stimulus, and finally giving rise to the all-or-none spike if the original stimulus is of sufficient strength. Figures 6 and 7 depict a similar train of events. The plateau reached quickly after the application of the conditioning pulse represents the increase in excitability due to the charging process of the membrane. The sudden further decrease in threshold occurring at 50 to 80 μ sec. marks the point of entry of the local potential. Since the conditioning pulse is subthreshold, no excitation occurs, but the threshold subsequently rises along an approximately exponential course. (The sigmoidal shape of the latter part of the experimental curve is due to the logarithmic abscissa.) In fact, if figures 6 and 7 are plotted with descending voltage values upward, the picture closely resembles results obtained with electrical recording of subthreshold excitatory events provided that the logarithmic abscissa is compensated for.

The time of about 50 to 80 μ sec. shown in figures 6 and 7 for the latency of the muscle local response agrees with the tentative estimate made by Hodgkin ('38) for the local-response latency in crab nerve. Although there was some un-

certainty about the exact value of the local response latency, Hodgkin definitely demonstrated that the charging time for the nerve membrane was of extremely short duration. The value of under 30 μ sec. which can be deduced from figures 6 and 7 for the muscle membrane charging time agrees substantially with Hodgkin's estimate for crab nerve.

It can now be emphasized that the β - α discontinuity occurs, in experiments with small electrodes, between 50 and 150 μ sec.; and the latency of the local response varies in small electrode experiments between 50 and 80 μ sec. Therefore, the pulse duration at which the discontinuity in the SDC occurs coincides approximately with the duration of the latent period of the local response. Both the above facts and the following reasoning indicate that the latency of the local response is responsible for the β excitability.

While it is known that nerve and muscle membranes possess appreciable capacitance, Katz ('37) and Rosenberg ('37) have shown that the time constant for the electrotonic charging process of the nerve membrane is significantly smaller than the excitation constant. Therefore, the capacity of the membrane cannot be the only factor governing excitation kinetics. Rushton ('37) showed on theoretical grounds that an electrotonic charging process followed by a local membrane response would give a simple, exponential SDC if it were assumed that, as soon as the membrane was charged to the critical value, the contribution of the local response occurred instantaneously, so that no matter how brief a stimulating pulse was used, excitation occurred via *cooperation* between the stimulating pulse and the local response. This investigation has shown that, at least with small electrodes, the above assumption is valid only for relatively long stimuli.

Although there is a 50 to 80 μ sec. latency in the local response, it has nevertheless been possible to cause excitation with a testing pulse of 5 μ sec. duration. This must mean that pulses of very short duration are able to set up the necessary conditions for a propagated response without the aid of a local response. That is to say, the local response does not participate

in attaining the threshold at short durations, although it is, of course, a factor in the subsequent growth of the potential which eventually becomes the spike. At longer durations, the stimulus is able to sum with the local response and therefore needs be of smaller intensity than if it alone had to supply all the energy necessary to trigger propagation. This is then the basis for a discontinuity in the muscle SDC. At very short durations (under about 100 μ sec.) the threshold for a propagated response is reached without the aid of the local response, and the kinetics of the process are governed solely by the membrane and stray circuit capacity. At about 50 to 150 μ sec., the threshold for excitation decreases markedly due to the fact that local response is now aiding the stimulating pulse in reaching threshold by adding some of the potential energy across the membrane to the excitation process. The kinetics of the latter process are governed by the time necessary for the local response to become propagating (Rushton's propagation constant, h). Therefore, a SDC taken of a tissue which manifests such characteristics would of necessity show a discontinuity at the point of entry of the local response due to the drop in stimulating threshold at that point. It is to be emphasized that this analysis shows that the β excitability is a process which is adequately explained by the simple *physical* condenser model. If, then, the β excitability is taken to represent the physical charging constant of the membrane, it is difficult to reconcile the value of between 20 and 30 μ sec. found here and the value of 8 msec. found by Katz ('48) for the time constant of the muscle membrane. Even though Katz' electrodes were much larger than those used in this investigation, the data of this paper indicate that the β excitability does not change with electrode size. Katz ('48), however, has demonstrated with direct recording the local response of the muscle fiber membrane.

Rushton's theoretical prediction that the SDC of a tissue displaying both a physical charging process and a local response should be exponential in nature applies at least approximately to the α SDC, and this prediction has been fully

verified. The β SDC is not predicted by Rushton's formulation due to his assumption of a local response with zero latent period.

The data of Rushton and Rashbass ('49) show that the sheath causes the excitation characteristics of myelinated nerve to differ from those of striated muscle. This fact may explain the reason that a β excitability has never been reported for myelinated nerve.

There are two complications to this explanation.

1. A discontinuity in the muscle SDC was found only in about 50% of the cases with curarized muscle although the discontinuity in the accommodation curve was always found.

2. Denervated sartorius muscles (fig. 8) did not show the initial plateau in accommodation experiments but behaved as if the rise of the local potential was much accelerated.

The explanation of the first point probably lies in the fact that accommodation experiments done in the early fall (fig. 7 A) show an entry of the local response 20 to 30 μ sec. later than experiments done in early winter (figs. 7 B and 7 C). This difference was consistent, although small. (Three experiments were done in September and three in January.) Nevertheless, since the shortest duration used for SDC measurement was 30 μ sec., a shift from 80 to 50 μ sec. in the point of local response appearance would result in only one or two points being noticeably displaced from the exponential α curve and would not be counted as significant enough a deviation to establish a β excitability at very short durations. Figures 4 C and 4 D are SDC which tend towards this type of behavior at very short durations.

The effect of denervation in accelerating the rise of the local response is probably a basic feature of the often observed supersensitivity of denervated structures. It is possible that experiments done on winter frogs show a decrease in latency of the local response due to the poor condition of these specimens and that denervation causes an even greater decrease in latency due to the poorer condition of the muscle after two weeks of denervation. In this regard it should be pointed out

that SDC's of denervated muscles never showed any discontinuities, and, therefore, the results are not incongruous with the suggestion that a significant decrease in local response latency would remove the β excitability from the range of experimental measurement available here.

The data on utilization times presented above can be used in conjunction with the results of the accommodation experiments of figures 6 to 8 to show that Hill's and Rashevsky's theories fail to agree with experimental results obtained by stimulation with small electrodes. Hill's λ estimated from figures 6 to 8 is about 1.0 msec. Utilization times determined here have ranged from 1.9 to 2.7 msec. However Hill's ('35a) theory predicts a utilization time of 0.33 msec. which is significantly shorter than any experimental value reported here. Rashevsky's theory predicts with the data given that accommodation is negligible. Figures 6, 7 and 8 contradict this prediction. Therefore, it must be concluded that neither Hill's nor Rashevsky's theories are in accord with experimental facts.

I wish to gratefully acknowledge the generous help and encouragement given me by Dr. C. L. Prosser during the undertaking of the above research.

SUMMARY

Strength-duration curves of single, isolated muscle fibers of the frog semitendinosus stimulated with large electrodes in solution display an α and a γ branch. The γ branch is eliminated by curare. The γ branch has an excitation constant (Hill's k) of about 0.3 msec. and the α branch a constant of 7.5 msec. Small electrode stimulation of curarized single muscle fibers stimulated in paraffin oil yields an α strength-duration curve with an excitation constant of about 0.17 msec. Curare does not eliminate a further discontinuity which occurs at durations of about 0.08 to 0.15 msec. This excitability has a constant of about 0.03 msec. and is considered identical to Lucas' β excitability. This excitability occurred in about

50% of experiments and had a seasonal correlation. Since direct measurement of excitability during a sub-threshold conditioning pulse disclosed a delayed entry of the local response at the same range of times in which the β - α discontinuity in the strength-duration curve occurred, it has been concluded that the discontinuity in the strength-duration curve is due to this delayed entry of the local response. Denervation shortened the latency of the local response entry.

All α , β and γ curves obtained were exponential in shape, although Hill's and Rashevsky's complete theories failed to agree with the experimental data. Blair's constant, C , was not significantly different from zero.

LITERATURE CITED

- BLAIR, H. A. 1941 Muscle excitability. *Biological Symposia*, 3: 51.
- COLE, K. S., AND H. J. CURTIS 1941 Membrane potential of the squid giant axon during current flow. *J. Gen. Physiol.*, 24: 551.
- DUBBERT, D. R., AND O. H. SCHMITT 1949 Tissue stimulators utilizing radio frequency coupling. *Rev. Sci. Instr.*, 20: 170.
- FISHER, R. H. 1950 *Statistical Methods for Research Workers*. Hafner, New York.
- GRUNDFEST, H. 1932 Excitability of the single fiber nerve-muscle complex. *J. Physiol.*, 76: 95.
- HILL, A. V. 1910 A new mathematical treatment of changes of ionic concentration in muscle and nerve under the action of electric currents with a theory as to their mode of action. *J. Physiol.*, 40: 190.
- 1935a Excitation and accommodation in nerve. *Proc. Roy. Soc.*, B119: 305.
- 1935b The strength-duration relation for electrical excitation of medullated nerve. *Proc. Roy. Soc.*, B119: 440.
- HODGKIN, A. L. 1938 The subthreshold potentials in a crustacean nerve fiber. *Proc. Roy. Soc.*, B126: 87.
- HODGKIN, A. L., AND W. A. H. RUSHTON 1946 The electrical constants of a crustacean nerve fiber. *Proc. Roy. Soc.*, B133: 444.
- JINNAKA, S., AND R. AZUMA 1922 Changes in the electrical excitability of the amphibian muscle fiber and discussion of A. V. Hill's excitation formula. *Proc. Roy. Soc.*, B94: 71.
- KATZ, B. 1937 Experimental evidence for a non-conducted response of nerve to subthreshold stimulation. *Proc. Roy. Soc.*, 124: 244.
- 1948 The electrical properties of the muscle-fiber membrane. *Proc. Roy. Soc.*, 135: 506.
- KUFFLER, S. W. 1945 Electrical excitability of nerve-muscle fiber preparations. *J. Neurophysiol.*, 8: 77.

- LAPICQUE, L. 1907 Recherches quantitatives sur l'excitation électrique des nerfs. *J. Physiol. Path. Gen.*, 9: 620.
- 1924 La Chronaxie en Fonction du Temps. Les presses universitaires de France, Paris.
- 1931 Has the muscular substance a longer chronaxie than the nervous substance? *J. Physiol.*, 73: 189.
- 1934 Neuromuscular isochronism and the chronological theory of curarization. *J. Physiol.*, 81: 113.
- LE FEVRE, P. 1950 Excitation characteristics of the squid axon. A test of excitatory theory in a case of rapid accommodation. *J. Gen. Physiol.*, 34: 19.
- LUCAS, K. 1906 On the optimal electric stimuli of normal and curarized muscle. *J. Physiol.*, 34: 372.
- 1906 On the optimal electric stimuli of muscle and nerve. *J. Physiol.*, 35: 372.
- 1907 The excitable substances of amphibian muscle. *J. Physiol.*, 36: 113.
- MONNIER, A. M. 1934 L'excitation Électrique des Tissus. Les presses universitaires de France, Paris.
- PROSSER, C. L., AND A. H. CHAMBERS 1938 Excitation of nerve fibers in the squid (*Loligo Pealii*). *J. Gen. Physiol.*, 21: 781.
- RAMSEY, R. W., AND S. F. STREET 1938 The alpha excitability of the local and propagated mechanical response in isolated single fibers. *J. Cell. and Comp. Physiol.*, 12: 361.
- RASHEVSKY, N. 1938 Mathematical Biophysics. University of Chicago Press, Chicago.
- ROSENBERG, H. 1937 Electrotonus and excitation in nerve. *Proc. Roy. Soc.*, 124: 210.
- RUSHTON, W. A. H. 1930 Excitable substances in the nerve-muscle complex. *J. Physiol.*, 70: 317.
- 1933 Lapicque's theory of curarization. *J. Physiol.*, 77: 337.
- 1935 The time factor in electrical excitation. *Biological Reviews*, 10: 1.
- 1937 Initiation of the propagated disturbance. *Proc. Roy. Soc.*, 124: 210.
- RUSHTON, W. A. H., AND C. RASHBASS 1949 The relation of structure to the spread of excitation in frog's sciatic trunk. *J. Physiol.*, 110: 110.
- WATTS, C. F. 1924 The effect of curari [*sic*] and denervation upon the electrical excitability of striated muscle. *J. Physiol.*, 59: 143.

IONIC MIGRATION IN ISOLATED NERVES

A. VAN HARREVELD AND F. E. RUSSELL

*William G. Kerkhoff Laboratories of the Biological Sciences,
California Institute of Technology, Pasadena*

ONE FIGURE

Cowan ('34) found that isolated crab nerves lose potassium to the surrounding sea-water. Arnett and Wilde ('41) observed that isolated frog nerves kept in oxygenated Ringer's solution also lose potassium. This loss was sustained during the first 2 to 5 hours after isolation. Fenn and Gerschman ('50) made a similar observation. A loss of potassium and a gain of sodium in isolated axons of the squid was found by Steinbach and Spiegelman ('43) and by Keynes and Lewis ('51). The same observation was made in *Sepia* axons (Keynes, '51; Keynes and Lewis, '51). Van Harreveld ('51) found that isolated rabbit nerves kept in oxygenated Ringer's solution lose potassium for the first few hours. From the 4th hour after isolation no loss was sustained.

Fenn and Gerschman ('50) considered the possibility that potassium is lost by diffusion from the cut ends of the nerve. They found however that tying off the cut ends did not prevent the loss. Van Harreveld ('51) considered the possibility that the potassium loss is due to the injury potential, which will cause a migration of sodium ions from the surrounding medium into the cut ends, and a release of potassium through the mantle of the nerve. Such a mechanism would be consistent with the observation that the loss of potassium in vertebrate nerves is incurred during the first hours after isolation since the injury potential tends to decrease and disappear during this period by a "sealing over" process (Gerard, '30; Lorente de N6, '47). The present investigation presents evidence in favor of such a mechanism of potassium loss.

METHODS

Part of the experiments were performed on frog nerves (*Rana catesbiana*). In these experiments a Ringer's solution was used containing 110 mM NaCl, 1.9 mM KCl, 1.1 mM CaCl_2 , 1.2 mM NaHCO_3 and 11 mM glucose. The solution was oxygenated by a mixture of 5% CO_2 in O_2 . In other experiments, nerves dissected out of the meropodite of one of the walking legs of the spiny lobster (*Panuliris interruptus*) were used. These nerves were bathed in an artificial sea-water containing 440 mM NaCl, 9 mM KCl, 9 mM CaCl_2 , 20 mM MgCl_2 . No glucose was added. For oxygenation commercial O_2 was used.

To determine the potassium content of the nerve, adhering fluid was removed with filter paper and the nerve weighed in a platinum crucible. The nerve was then dried at about 100°C . for 24 hours, weighed again and ashed at 500°C . The ash was dissolved in 1 cm^3 5% acetic acid and transferred quantitatively with doubly glass distilled water to a 5 cm^3 volumetric flask. On this fluid the potassium and sodium determinations were performed using a Beckman flame spectrophotometer. The potassium content of the nerve was computed on the basis of wet and dry weight and expressed in millimoles per kilogram. The percentage changes were rounded to the nearest 0.5%.

RESULTS

A. *Potassium loss in frog nerve.* A piece of frog's sciatic nerve 3 cm long was isolated, weighed and dried, to serve as control. Another 3 cm length of nerve was taken from the heterolateral sciatic. This was kept for two hours at 18°C . in Ringer's solution through which 5% CO_2 in O_2 was bubbled. The nerve was then weighed and dried. The results of the K determinations of control- and treated nerves in 10 experiments are presented in table 1. The experimental nerves lost 8.5% of their K based on the wet weight figures, which is significant at the 5% level ($t=2.9$). Based on the dry weights the loss is 11.5%. This is significant on the 1% level ($t=5.0$).

TABLE 1
Potassium losses in isolated frog and lobster nerves (3 cm)

EXPERI- MENTAL ANIMAL	NUMBER OF EXPERIMENTS	CONTROL (mM/kg)		TREATMENT	TREATED (mM/kg) % DIFFERENCE	
		Wet	Dry		Wet	Dry
Frog	10	28.4	120	Control nerve no treatment.	26.0	106
				Experimental nerve 2 hours in Ringer's with 0.2% glucose and 5% CO ₂ in O ₂ .	-8.5 (± 2.9%)	-11.5 (± 2.3%)
Lobster	10	266	1805	Control nerve no treatment.	193	1470
				Experimental nerve 1 hour in sea-water with O ₂ .	-27.5% (± 2.4%)	-18.5% (± 2.2%)

TABLE 2

Potassium loss and sodium gain in isolated lobster nerve (3 cm). The control nerve was not treated. The experimental nerve was kept for 1 hour in oxygenated sea-water. It was then divided in three equal parts

ION INVESTI- GATED	NUMBER OF EXPERIMENTS	CONTROL (mM/kg)		TREATED (mM/kg) % DIFFERENCE					
		Wet	Dry	Middle			Ends		
				Wet	Dry		Wet	Dry	Total
K	10	271	1914	251	1940		177	1404	1577
				-7.5% (± 3.1%)	+1% (± 2.6%)		-34.5% (± 1.8%)	-26.5% (± 2.6%)	-17.5% (± 2.3%)
Na	10	152	1097	156	1130		251	1960	1670
				+2.5% (± 2.1%)	+3% (± 2.7%)		+65.5% (± 7.2%)	+78.5% (± 9.3%)	+52.5% (± 6.5%)

The greater K loss on the basis of the dry weights may indicate a water loss of the experimental nerves while in the Ringer's solution. In agreement with this it was found that the percentage of solids as computed from wet and dry weights was greater for the experimental than for the control nerves. The potassium loss in frog nerve is quite similar to that in rabbit nerve under similar conditions (van Harreveld, '51).

B. *Potassium loss in lobster nerve.* A similar experiment as described for frog nerve was carried out with 3 cm lengths of nerve taken from the meropodite of walking legs of lobsters. The nerve was left for only one hour in sea-water (18°C.) through which O₂ was bubbled. The results of 10 experiments are shown in table 1. The K loss computed on the basis of the wet weights is 27.5%, on the basis of the dry weights 18.5%. This loss is highly significant ($t=11.5$ and 8.4 respectively). The K loss of lobster nerves was smaller when computed on the basis of the dry weights than on the basis of the wet weights. This would indicate that the experimental nerves had taken up water. A computation of the percentage of solids in experimental and control nerves supported this conclusion. Although the lobster nerves were isolated for one hour instead of two hours as in the case of the frog nerves, the potassium loss is considerably larger.

C. *The distribution of the potassium loss over the nerve.* If the K loss is a consequence of the injury potential which causes a migration of sodium ions into the cut ends, and a loss of potassium ions through the mantle, then a decrease in the K concentration of the nerve adjacent to the cut end must be expected. To test this a 3 cm length of lobster nerve was kept for one hour in oxygenated sea-water at 18°C. as described before. It was then divided into three equal pieces. The middle third of the nerve was weighed, dried and ashed separately; the end pieces were treated together. The K content of the end and of the middle pieces was compared with that of a 3 cm section of lobster nerve taken from the contralateral leg and weighed and dried immediately after dissection. The results

are shown in table 2. Based on the wet weights the middle pieces lost 7.5% which is not significant at the 1% level ($t=2.4$); the end pieces however lost 34.5% of their K. This loss is highly significant ($t=19.2$). Based on the dry weights the middle pieces gained 1% which is not significant ($t=0.4$), but the end pieces lost 26.5%, a highly significant loss ($t=10.2$). A comparison of the changes in middle- and end pieces shows the differences between the groups to also be highly significant ($t=7.6$ and 7.4 respectively for the figures based on wet and dry weights). When the K loss of the entire nerve is computed from the potassium losses and weights of the middle- and end pieces, a deficit of 26% is found based on the wet weights and of 17.5% based on the dry weight figures. These figures are similar to those found by the direct determination of the potassium loss of a 3 cm piece of lobster nerve bathed in sea-water for one hour as shown in table 1. Again the loss based on the dry weights is smaller than that based on the wet weights, indicating that the nerve took up water during the experimental period.

D. *Changes in the sodium concentration of nerve.* If the loss of the K in lobster nerve, kept in sea-water, is due to the activity of the resting potential, then an increase in the sodium concentration of the nerve adjacent to the cut end can be expected. In a series of experiments 3 cm sections of lobster nerves were kept in oxygenated sea-water for one hour. They were then cut in three equal pieces. The sodium content of the middle- and end pieces was compared with that of a 3 cm length of nerve taken from the contralateral walking leg and weighed and dried without treatment. The results are shown in table 2. Based on the wet weights the middle piece of the experimental nerve showed an increase of 2.5% which is not significant ($t=1.3$). The Na concentration in the end pieces, however, increased 65.5%, a highly significant figure ($t=9.1$). The figures based on the dry weights revealed an increase of 3% for the middle piece (which is not significant, $t=1.2$). The end pieces increased 78.5%, which is highly significant ($t=8.3$).

The differences in gain of middle- and end pieces is highly significant statistically ($t = 11.2$ for both the wet and dry figures). The greater gain of Na when computed on the basis of the dry weights indicates that the lobster nerves, as in the experiments described above, take up water while being kept in sea-water. The gain in Na can be computed for the entire 3 cm length of the nerve. It was 44% based on the wet weights and 52.5% based on the dry weights (both are highly significant, $t = 7.7$ and 8.1 respectively).

E. *The locus of the potassium loss of nerve.* The findings of a potassium loss and a sodium gain near the cut ends of a nerve is compatible with either of two possibilities; (a) with the concept that the K loss is due to the action of the injury potential, and (b) with the possibility that K diffuses out, and Na diffuses into the cut ends of the nerve. To determine the locus of the K loss, a 5 cm section of lobster nerve was embedded in 5% gelatin in sea-water in the center of a tube of 5 mm in diameter. Since the gelatin melts at about 20°C. the lobster nerve need not be exposed to an unphysiologically elevated temperature. After cooling, the nerve with the surrounding gelatin was removed from the tube. A 3 cm piece of the nerve was isolated by two razor blade cuts through the nerve and its gelatin covering. On the two cut surfaces were placed 1 cm rods of 5% gelatin in sea-water of the same diameter as the gelatin covering of the nerve. The experimental setup is schematized in figure 1. The nerve embedded in gelatin was maintained at 18°C. for one hour. The oxygenation of the nerve is probably impaired but not prevented by the gelatin covering. Since lobster nerve is rather insensitive to oxygen lack (Wright, '47) there is no reason to believe that this will damage the nerve, and indeed seemingly normal action potentials have been led off from lobster nerves, after having been embedded for an hour in gelatin. At the end of the hour the gelatin rods approximated to the ends of the embedded nerve were transferred to 5 cm³ volumetric flasks and weighed. The embedded nerve was cut in three equal (1 cm) pieces. The nerve was then pulled out of the gelatin and the three resulting

cylinders of gelatin were transferred to 5 cm³ volumetric flasks and weighed. The gelatin was dissolved in warm doubly glass distilled water and the volume brought to 5 cm³. An untreated gelatin control rod of the same composition and size was also made up to 5 cm³ in a volumetric flask. The K content of the 6

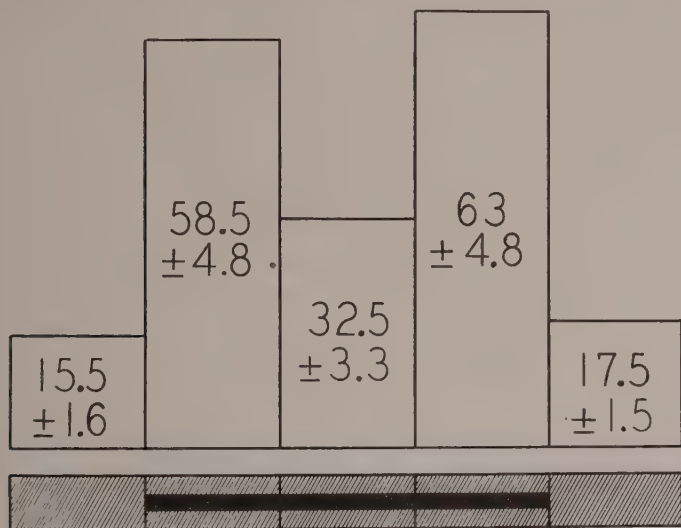


Fig. 1 The lower part of the figure shows schematically a 3 cm piece of lobster nerve (black) embedded in gelatin (striped). The graph in the upper part of the figure shows the release of potassium (in micrograms) into the 5 samples of gelatin surrounding and approximating the nerve. The heights of the blocks are proportional with the amount of potassium released in the gelatin samples. The figures are averages of 10 experiments. The standard errors are indicated.

flasks was determined. The K concentration of all the gelatin samples surrounding and approximating the nerve had increased as compared with the control.¹

From the K concentration of the gelatin samples and their weights, the gain in K of the various samples contacting the nerve can be computed. The results of 10 experiments are shown in figure 1. The gelatin surrounding the end pieces of

¹ The sea-water used contained 9 mM potassium/liter. The K concentration of the 5% gelatin in sea-water solution was 13 mM/liter. Part of this excess potassium may not be ionized.

nerve gained the greatest amount of K (58.5 and 63 μg). The gelatin surrounding the middle piece gained less (32.5 μg), and the gelatin rods approximated to the end of the nerve gained least (15.5 and 17.5 μg). The statistical treatment of these figures shows that the gain in all the gelatin samples is significant at the 1% level (t —in all instances around 10). The differences between K gains in the gelatin from around the middle piece, and in that from around the end pieces of the nerve, and of the gelatin rods fixed at the ends of the nerve, are all significant at the 1% level.

In a few experiments lobster nerves were embedded exactly as described above, with one exception, namely, that the gelatin samples were collected within one minute instead of an hour after the preparation was completed: The rods approximated to the ends of the nerve showed the greatest gain in potassium (8–9 μg). The gelatin surrounding the end pieces of the nerve gained somewhat less, and the gelatin surrounding the middle piece the least (about 4 μg). The gain in K of the rods must be due to their contact with the ends of the nerve mashed by the cutting. Since the gain an hour later has only about doubled (fig. 1) it may be concluded that very little potassium diffuses from the gelatin surrounding the nerve near the cut ends into the rods approximated to these ends. The gain in K of the gelatin surrounding the nerve may be due at least in part to the ionic migration caused by the injury potential. It requires from 4 to 5 minutes to embed the nerve, allow the gelatin to solidify, cut a 3 cm length of nerve and approximate the gelatin rods to the nerve ends. During this time the nerve will lose K through its mantle due to the injury potential.

DISCUSSION

The potassium and sodium concentrations based on the dry weights are independent of changes in the water content of the nerves. These figures therefore form a more reliable indicator of changes in ionic concentration of the nerves than figures based on the wet weights.

From his discussion of the currents and potentials in injured frog nerve, Lorente de Nó ('47) concluded that the contributions of successive membrane sections to the total injury current decrease with increasing distances from the injured point. The distribution of the injury potential along a transected frog nerve showed that these contributions become negligible beyond 1.5 cm from the injured region. The determination of the "space constant" (distance over which an electronic potential in the extrapolar region falls by a factor $1/e$) of sepia axons by Weidmann ('51) would indicate that for this species the contributions of the membrane to the injury potential become negligible at a somewhat greater distance from the injured region than the value found for frog nerve.

Potassium is the most abundant cation in the axoplasm. The nature of the anions is not completely known. Silber ('41) found that aspartic acid (and possibly other amino acids) accounts for part of the anions in the axoplasm of lobster nerve. Such organic ions are heavier than potassium, and have a smaller mobility. It would therefore seem that in the axoplasm a considerable part of the current is carried by the K ions. Since the cut place is negative with respect to the mantle of the fiber, sodium ions will enter the axoplasm at this region from the surrounding medium where they are the most abundant cation. In order to maintain electrical neutrality this will necessitate the release of K ions through the mantle of the fiber.

In the above concept the potassium released is proportional to the current through the membrane. Since the injury current is the largest through the sections of the membrane close to the injury and decreases with distance from the cut ends, the loss of potassium through the mantle of the nerve fiber should be the greatest near the cut ends. The observed release of potassium by isolated lobster nerve into the surrounding gelatin (fig. 1) is in agreement with this thesis. The potassium loss of the end pieces is almost twice that of the middle piece. The large release of K into the gelatin covering the end pieces of

the nerve and the small gain in K of the end rods becomes a strong argument against the loss of potassium by diffusion from the cut ends as was considered by Fenn and Gerschman ('50).

The middle third of the isolated nerve released an average of 32.5 μ g of potassium in one hour into the surrounding gelatin. Since the weights of the middle pieces average about 20 mg this would correspond to a release of 40 mM per kilogram. If this K was exchanged for Na such a loss would be easily detectable with the methods used. However, no significant loss of potassium or gain of sodium in that section of the nerve was observed (table 2). This is in agreement with the proposed mechanism of the potassium loss in isolated nerve, for the K lost through the mantle of the fibers in this nerve section is replenished by potassium which migrates in from the end pieces of the nerve.

It was found that based on the dry weights the end pieces of lobster nerves lost 510 mM/kg potassium and gained 863 mM/kg sodium (table 2). One would expect the gain in Na of the end pieces to be equivalent with the loss in K. However, it was observed that lobster nerves gained weight while being in sea-water. It seems possible that this weight increase was not due exclusively to an osmotic gain of water, but that the damaged ends of the nerve store some of the sea-water with its high sodium chloride concentration.

Since the changes in K and Na concentration occur only near the cut ends of the nerve, the length of the isolated piece of nerve is of importance when determining such changes in the entire nerve. It is for this reason that a standard length of 3 cm was adopted in this investigation.

The above observations were made on nerve that had been isolated for a relatively short period. With longer periods of isolation the situation may change considerably. The nerve in the immediate vicinity of the cut may become depolarized. The injury potential may decrease due to the "sealing over" processes at the cut ends (Gerard, '30; Cowan, '34; Lorente de

Nó, '47). The original sharp concentration gradients of the sodium and potassium ions in the regions close to the cut ends will become more gradual due to diffusion. An active "sodium pump" may remove some of the sodium from the axoplasm. These factors make it difficult to estimate how far the sodium ions will penetrate into the axoplasm during a given time.

Many authors have observed a loss of potassium and a gain of sodium in isolated nerves. Fenn and Gerschman ('50) considered the possibility of a loss of potassium through the cut ends of the nerve. This is not supported by the present experiments. Steinbach and Spiegelman ('43), Keynes ('51) and Keynes and Lewis ('51) considered as the only mechanism an exchange of sodium for potassium through the membrane. To what extent, if any, the ion transport due to the injury current has contributed to these results is difficult to estimate. Much depends on the duration of the injury current flow, the length of the isolated nerve, and on the region from which the sample was taken.

SUMMARY

Sections of frog nerve, 3 cm long, lost 11.5% of their potassium when bathed for two hours in oxygenated Ringer's solution containing glucose. Pieces of lobster nerve of the same length lost 18.5% of their potassium when kept for one hour in oxygenated sea-water. The middle third of such a 3 cm piece of lobster nerve did not significantly change in potassium and sodium concentration. The thirds adjoining the cut ends lost 26.5% of their potassium and increased 78.5% in sodium. It could be shown that lobster nerve releases potassium over its entire length, also the middle third of the isolated nerve which did not change in potassium concentration when kept for an hour in sea-water. The potassium lost by this section of the nerve was replenished from the potassium in the region near the cut ends. These observations support the thesis that the injury potential causes a migration of sodium ions into the axoplasm through the cut ends of the nerve and a release of potassium ions through the mantle of the nerve fibers.

LITERATURE CITED

- ARNETT, V., AND W. S. WILDE 1941 Potassium and water changes in excised nerve on stimulation. *J. Neurophysiol.*, *4*: 572-577.
- COWAN, S. L. 1934 The action of potassium and other ions on the injury potential and action current in *Maia* nerve. *Proc. roy. Soc. B.*, *115*: 216-260.
- FENN, W. O., AND R. GERSCHMAN 1950 The loss of potassium from frog nerves in anoxia and other conditions. *J. Gen. Physiol.*, *33*: 195-203.
- GERARD, R. W. 1930 The response of nerve to oxygen lack. *Am. J. Physiol.*, *92*: 498-541.
- KEYNES, R. D. 1951 The ionic movements during nervous activity. *J. Physiol.*, *114*: 119-150.
- KEYNES, R. D., AND P. R. LEWIS 1951 The sodium and potassium content of cephalopod nerve fibres. *J. Physiol.*, *114*: 151-182.
- LORENTE DE NÓ, R. 1947 A study of nerve physiology. Studies from the Rockefeller Institute for Medical Research, *131*: 1-9.
- SILVER, R. H. 1941 The free amino acids of lobster nerve. *J. Cell. and Comp. Physiol.*, *18*: 21-30.
- STEINBACH, H. B., AND S. SPIEGELMAN 1943 The sodium and potassium balance in squid nerve axoplasm. *J. Cell. and Comp. Physiol.*, *22*: 187-196.
- VAN HARREVELD, A. 1951 Asphyxial potassium loss of mammalian nerve. *J. Cell. and Comp. Physiol.*, *38*: 199-206.
- WEIDMANN, S. 1951 Electrical characteristics of sepia axons. *J. Physiol.*, *114*: 372-381.
- WRIGHT, E. B. 1946 A comparative study of the effects of oxygen lack on peripheral nerve. *Am. J. Physiol.*, *147*: 78-89.

THE PROPAGATION OF IMPULSES IN MYELINATED AXONS

A. ROSENBLUETH, J. GARCÍA RAMOS AND R. MILEDI

Department of Physiology of the Instituto N. de Cardiología of Mexico

SIX FIGURES

The theory of saltatory conduction in myelinated axons (Lillie, '25) has recently received support from many observations (see for references Hodgkin, '51), although dissenting opinions have also been expressed (Lorente de Nó, '47; Schneider, '49; Laporte, '51; Wolfgram and van Harreveld, '52). A critical appraisal of the arguments presented in its favor reveals that the majority of the arguments adduced are indirect, i.e., that they are compatible with the theory, but that they do not prove it. The present study was undertaken with the purpose of subjecting the theory to more direct tests than those that have been used previously.

METHOD

Several myelinated axons were studied. In a few experiments the nerves of the lateral line of the carp were tested, namely, the nerves studied by Laporte ('51). According to Kynaston Thomas and Young ('49) the internodal distances in the large fibers of these nerves are relatively great. In the species we obtained, however, these distances were no greater than those found in frog axons; in addition the amplitude of the action potentials was quite small. For these reasons the study of these fibers was not pursued.

In other observations the fibers from the Pacinian corpuscles of the cat's mesentery were dissected. The advantage of this preparation is that in many instances a single fiber runs isolated for several millimeters until it joins a

neighboring nerve trunk. The internodal distances in these fibers varied between 0.6 and 1.4 mm (average 1.0 mm).

In order to observe single fibers included in a small nerve trunk, one of the fine cutaneous branches to the thigh of the 7th spinal nerve of the frog was dissected, maintaining its connections to the nerve and cutting all the other branches. By selecting the stimuli applied to the nerve it was usually possible to study the fiber in the branch with the lowest threshold. The identification of the records from a single fiber is as a rule easily made on the basis of the pattern of the spike and of its all-or-nothing character. In the frogs used, the internodal distances of the large fibers in the cutaneous branches in question varied between 0.8 and 1.8 mm (average 1.2 mm). The diameter of the branches varied between 80 and 100 μ .

In some instances single fibers were isolated from different nerve trunks of frogs, following the technique described by Tasaki ('39). Unfortunately these fibers deteriorated promptly when placed in mineral oil. For this reason, notwithstanding the ostensible advantage inherent to the use of isolated single fibers that yield larger electric responses, the majority of the observations were carried out on the cutaneous nerve trunks mentioned above.

The nerves were placed on platinum wire electrodes in a mineral oil bath. In addition to several fixed electrodes, to the two end ones of which the nerves were tied by fine silk threads, a movable holder was used, which supported one to three fine electrodes (diameter about 0.1 mm). The movement of these electrodes was controlled by the micrometers of a microscope mechanical stage and was observed through a dissecting microscope.

The fibers were stimulated from 5 to 25 times per second by rectangular pulses applied to two of the fixed electrodes. The stimulator used was a Grass Model S4A unit; it controlled and synchronized the sweep circuit of the cathode-ray oscillograph. The pulses were passed through a Grass stimulus-isolation unit, to eliminate any contact with ground. The

nerves were grounded usually between the stimulating and the recording electrodes.

The electric responses of the fibers were led to a cathode-follower input stage, from this to a 5-stage resistance-capacity coupled amplifier, and finally to a cathode-ray oscillograph. The over-all non-distorted frequency range was from 2 to 10,000 c.p.s. One or two independent similar recording channels were employed.

RESULTS

A. The latency of the monophasic action potentials. In these observations the stimulating electrodes were in a fixed position, as well as the distal recording lead on dead tissue. The proximal recording lead was one of the movable electrodes. It was displaced along the fiber or trunk by steps of 0.2 or 0.3 mm. The corresponding responses were photographed and were later studied after adequate enlargement.

For reasons that will be given in the discussion, the point selected to measure the changes in latency was that corresponding to 50% of the maximum amplitude, on the ascending branch of the spike potential. Figure 1 illustrates typical results. It is clear that the scatter in the measured points does not allow to discriminate whether the latency increases in a continuous manner with conduction distance, or whether the increase is discontinuous, with breaks corresponding to the nodes (see the two curves drawn through the circles in fig. 1).

In another series of observations two monophasic records were taken simultaneously, from two of the movable electrodes to a common lead on the crushed end. The difference in the latencies of the two spike potentials was plotted against the conduction distance along the fiber. As shown in figure 1 (lower tracing) these differences do not show discontinuities, but there are oscillations which may be attributable to the presence of nodes.

B. The amplitude of the monophasic spike potentials. Figures 2 and 3 illustrate the amplitude of the spike potentials

recorded from different points along a fiber. In all the observations made, this amplitude did not exhibit cyclic oscillations attributable to the presence of the nodes. It remained practically constant, declining only in a gradual manner when the lead on intact tissue approached the damaged end of the fiber.

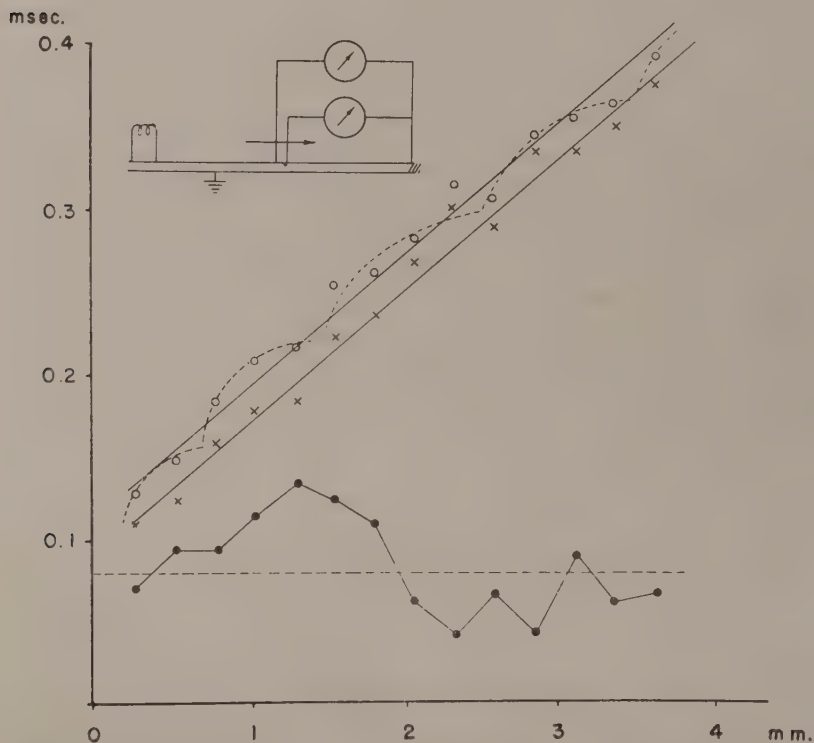


Fig. 1 Latencies of the monophasic responses of a single fiber with increasing conduction distance. Single fiber in a frog cutaneous strand. Stimuli applied to the 7th spinal nerve. Ordinates: time in milliseconds. Abscissae: distances along the fiber in millimeters; the zero point is arbitrary. Circles and crosses: latencies of the responses from two recording electrodes displaced along the strand with a constant distance of 0.27 mm; these latencies were measured from the beginning of the stimulus artifact to 50% of the maximum amplitude on the ascending branch of the corresponding spikes. Dots: differences in the latencies between each pair of records, multiplied by 4. The dotted line shows the mean difference between the two upper lines, multiplied by 4.

C. The amplitude of the diphasic records taken in different positions along the fibers. In these observations the stimulating electrodes were kept in a fixed position. A pair of recording electrodes with a constant interelectrode distance of 0.2 to 0.5 mm was moved by steps of 0.1 to 0.3 mm along the fiber or trunk.

The results were not uniform, because, as will be indicated in the discussion, several factors influence the amplitude of the diphasic records. The data, however, justify the statement that periodically recurring oscillations may

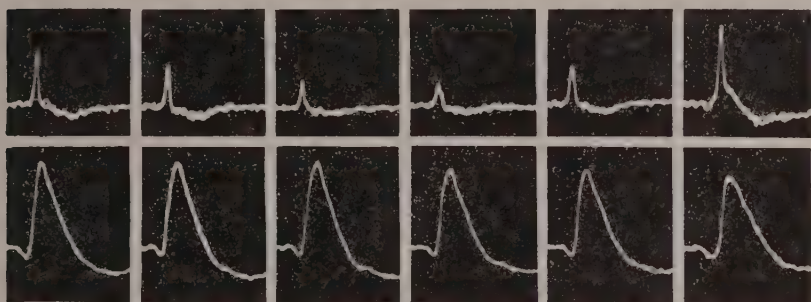


Fig. 2 Diphasic and monophasic records taken from different points along an internodal segment. Single fiber in a cutaneous strand of the frog. Two electrodes with a constant distance of 0.4 mm were moved by steps of 0.3 mm along the strand. The upper tracings are records from these two electrodes. The lower tracings (with less amplification) are the records from the proximal electrode in this pair to another placed on the crushed peripheral end of the strand.

appear, that are due to the position of the electrodes with regard to the nodes. Thus, in the frog nerves cyclical oscillations appeared, whose spatial period was within the range of the internodal distance measured in the large myelinated fibers. And, in favorable instances, in the single fibers of the Pacinian corpuscles this period coincided accurately with the internodal distances measured in the same fibers after fixation and staining with osmic acid. Figures 2 and 3 illustrate characteristic examples.

D. The amplitude and duration of the diphasic records as a function of the recording interelectrode distance. In

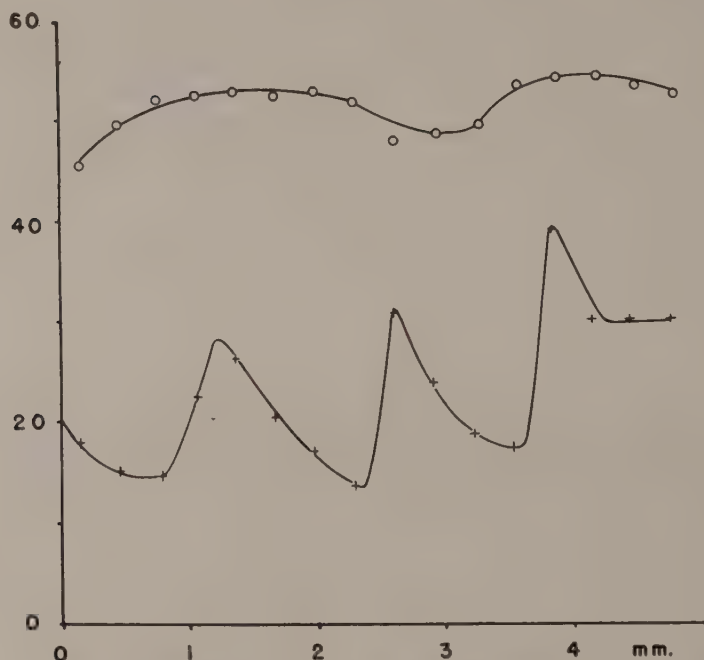


Fig. 3 Records taken as in figure 2. Abseissae: distance along the strand in millimeters. Ordinates: maximal amplitudes in conventional units. Circles: monophasic responses. Crosses: diphasic responses.

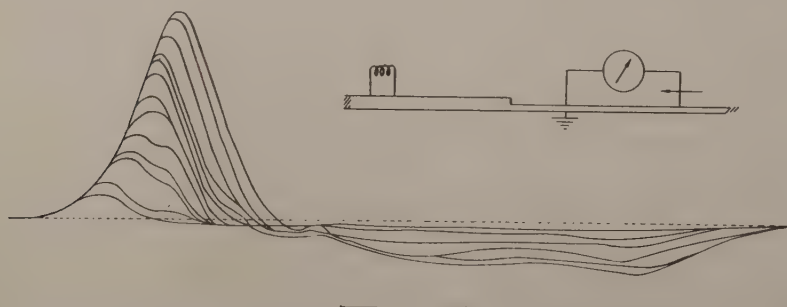


Fig. 4 Diphasic records with variable recording interelectrode distance. Single fiber in a cutaneous strand of a frog. The proximal lead was kept fixed, and the distal (see inset diagram) was moved by steps of 0.3 mm so that the recording distance varied from 3.8 to 0.2 mm. The drawings show the superimposed corresponding records. Time calibration: 0.1 msec.

these observations, with fixed stimulating electrodes and also a fixed proximal recording lead, the distal recording electrode was moved away or toward the proximal lead by steps of 0.1 to 0.3 mm. Within a range that depends on the duration of the rising branch of the spike potential and on the conduction velocity of the impulses, the recording interelectrode distance markedly modifies the amplitude and duration of the first phase of the diphasic records. As the interelectrode distance increases, both the amplitude and the duration also increase. Figure 4 illustrates a characteristic group of records. It is interesting to note that the increase of amplitude with distance is approximately uniform, i.e., that it does not show any abrupt changes.

E. The derivative of the diphasic records with respect to distance. As pointed out by Huxley and Stämpfli ('49) and by Hodgkin ('51) this derivative is of importance because it yields information on the inflow or outflow of current through the axon membrane. A direct record of this derivative was obtained by placing three movable recording electrodes *a*, *b* and *c*, with short interelectrode distances, e.g., 0.3 to 0.5 mm (see diagram in fig. 5). The distance between *a* and *b* and that between *b* and *c* was deemed equal when each pair yielded diphasic records of equal amplitude when placed in the same position on the nerves. The three electrodes were on the same holder, so that these distances were preserved when they were moved along the nerve.

The space derivative corresponding to the point in contact with electrode *b* can be obtained by summing algebraically the record from *a* to *b* to that from *b* to *c*. In some observations this summation was obtained by connecting *a* and *c* together and leading from this connection to *b*. In later experiments a summation that preserved the independence of the electrodes on the nerves was obtained as follows. The output of the preamplifiers used is a cathode-follower stage. This output from the two amplifiers connected with *a* and *b*, and *b* and *c*, respectively, was mixed as above and led to the input condensers of a single power amplifier.

The main purpose of these experiments was to explore the membrane current in the internodal regions. This requires observations with the three electrodes placed in these regions, which in turn requires relatively short interelectrode distances. But the difference between two diphasic records from small contiguous regions is quite small, so small that in many instances it is hardly above the noise level of the amplifiers.

For these reasons, of a long series of experiments only a few were considered satisfactory. Figure 5 illustrates one of them. The records show cyclic oscillations of amplitude, but remain diphasic in all the positions of the electrodes. They thus indicate that an initial outflow is followed by a later inflow of current through the axon membrane and myelin sheath.

DISCUSSION

1. *The latency of the monophasic spike potential.* Laporte's ('51) argument against saltatory propagation is based on the inference that the latency of the spike potentials increases uniformly with conduction distance and does not show any discontinuities corresponding to the nodes. This inference is at variance with the data reported by Huxley and Stämpfli ('49), who found, that the latency of the first phase of diphasic records increased discontinuously with increasing distance, and that the discontinuities corresponded

Fig. 5 Records of the diphasic character of the membrane current along an internodal stretch. Single fiber in a cutaneous strand of the frog. Three electrodes spaced 0.4 mm apart (see diagram) were moved by steps of 0.1 mm along the strand. The drawings show the records obtained by summing algebraically the responses between electrodes *a* and *b* to those between electrodes *b* and *c*, through the combination of the output of the two corresponding amplifiers. The horizontal lines that take the place of two of the records indicate that the signals were not clearly identifiable from the noise level. The middle line is the time calibration of the records, with 0.1 msec. intervals.

The diagram at the bottom is drawn to scale to show the size of the electrodes as compared to the average internodal distance, and diameters of the fibers and strands.

Fig. 6 Diagram used to show that the propagation of impulses is continuous. For explanation see text.

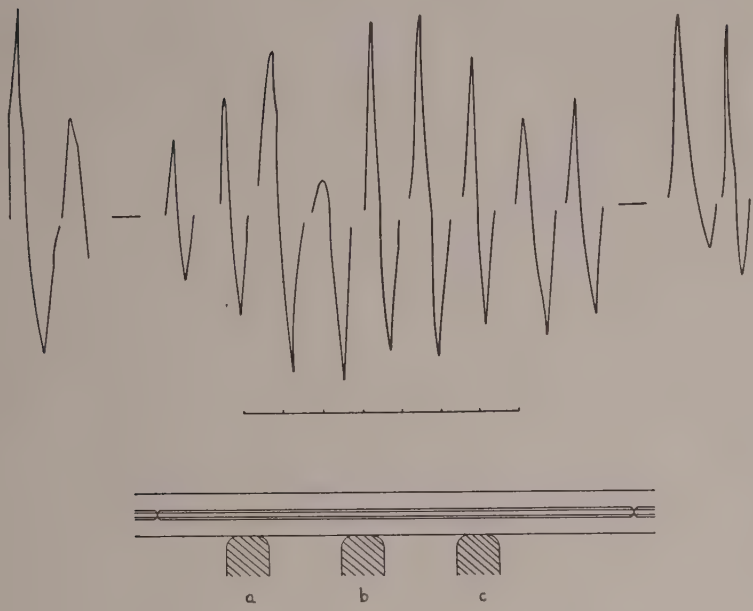


Figure 5

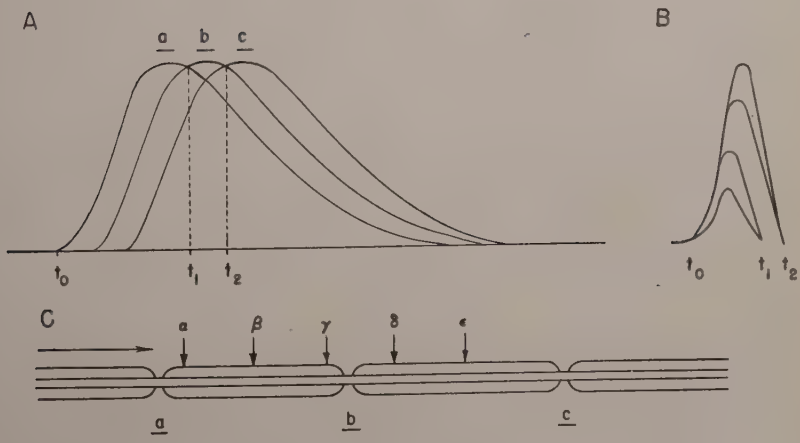


Figure 6

to the location of the nodes. Laporte's inference is based on inadequate data; his records show an important scatter, too great to allow a discrimination between the two opposed views in question. On the other hand, the present data prove that the interpretation of Huxley and Stämpfli is not acceptable, as will be shown below.

First, it is important to stress that the measurements of latencies are subject to several sources of error, which, although not important for the determination of conduction velocities over relatively long stretches of nerves, become quite significant when the differences of latency to be determined are in the order of small fractions of a millisecond. Laporte used sometimes the beginning of the spike potential, sometimes the peak, for his measurements. Both these points are difficult to determine, since the derivative of the spike with respect to time changes very slowly in those regions. The point whose latency can be determined with greatest accuracy is the inflection point on the rising branch, where the derivative is greatest (see Rosenblueth, Wiener, Pitts and García Ramos, '48). For this reason we selected in the present study the point with half the maximum amplitude, which is near the inflection point and is more readily measured.

As shown in figure 1, however, even if the point selected can be accurately measured, the scatter of the values found does not allow to discriminate whether the latencies vary continuously or discontinuously. In addition to amplifier noise, a reason for the scatter found is that the latency at a given point is not only determined by the conduction distance, but also by the duration of the initiation delay, which may vary importantly, especially, if the stimuli are only slightly above threshold (see Blair and Erlanger, '33, '36; Lorente de Nó, '47; Rosenblueth and Luco, '50).

The changes in the amplitude and duration of the diphasic records taken with variable interelectrode distance, illustrated in figure 4, show conclusively that the latency of the monophasic spike potential increases gradually and uniformly

with the conduction distance. A diphasic record can always be interpreted as the algebraic sum of two monophasic records, because this interpretation only states that Kirchhoff's e.m.f. law applies to nerves, much as it applies to physical circuits (see Rosenblueth, Daughaday and Bond, '43). The rising branch of the diphasic records shows the development of the spike potential at the region in contact with the proximal electrode. The later drop is due to the development of a similar spike at the level of the second electrode. Thus, the records in figure 4 show that the spike potential develops gradually later at the region in contact with the distal electrode when this electrode is gradually moved away from the proximal one.

This conclusion is supported by the measurements of latency shown in figure 1. There is always an approximately constant difference of latency between the spike potentials recorded from two electrodes less than 1 mm apart, regardless of the position of these electrodes with respect to the nodes. Given the conduction velocity of the fiber, this difference agrees with the interelectrode, not with the internodal distance. If the latency of the monophasic responses were constant through any given internodal space the graph of the differences would have yielded mainly zero values with sudden jumps to positive values when a node lay between the two leads on intact tissue.

As mentioned above, Huxley and Stämpfli ('49) reported that the latency of the first phase of diphasic responses remained constant along the internodal stretches, and only increased by steps when a nodal length was added to the conduction distance. We attribute this discrepancy between their results and ours to differences in the method of recording. They recorded from two pools of Ringer's solution in which the ends of the nerves were immersed, joined by a fine capillary channel through which an isolated single fiber was threaded. In these conditions, it is likely that the records correspond mainly to the events taking place at the nodes in the pools in the close neighborhood of the ends of the capillary channel, since their diffuse electrodes always

made contact with these nodes. The position of the capillary in the internodal stretch would then have no influence on the latency of the nodal responses. It is important to mention, however, that using essentially the same method, Hodler, Stämpfli and Tasaki ('52) found a gradual increase of latency as the recording stretch moved along an internodal segment.

2. *Formulation of the saltatory theories.* It is interesting to note that the majority of the studies on the problem do not formulate precisely what is meant by saltatory propagation, and what is the mechanism of the propagation. Saltatory usually means discontinuous, but there are several possibilities of discontinuity.

Erlanger and Blair ('34) suggested that the myelinated axons are constituted by unitary segments, bounded longitudinally at the nodes. Each segment would become active and stimulate the following one. Propagation velocity would then depend on the lag of transmission across nodes. This view dismisses the role of eddy currents in propagation, which is strongly supported by the evidence available. We shall, therefore, not consider it further in this discussion.

According to Huxley and Stämpfli ('49) activity occurs exclusively at the nodes, i.e., the internodal stretches are inactive. The same view was adopted by Hodgkin ('51). Tasaki at first upheld the same hypothesis ('39), but later has suggested that propagation is saltatory with respect to space but not with respect to time, that is, that there are spatial discontinuities in the physiologically active regions, namely, the nodes, but that the process of propagation is continuous in time (see Hodler, Stämpfli and Tasaki, '52).

3. *The insulating properties of the myelin sheath.* Although no direct measurements are available, it is quite likely that the resistivity of myelin is very high. The view held originally by Tasaki ('39), that the myelin sheath must be regarded as a practically perfect electric insulator, however, is no longer tenable, since currents may flow across this sheath (Huxley and Stämpfli, '49; Hodler, Stämpfli and Tasaki, '52; and the present study).

4. *Arguments in favor of saltatory propagation.* The majority of the arguments that have been proposed in favor of the theory of saltatory propagation are indirect and are not incompatible with the theory of continuous propagation, as follows.

It has been proved conclusively that several features of myelinated axons exhibit cyclic oscillations when explored at different points along the fibers, and that the spatial period of these oscillations corresponds to the internodal distances. Thus, the threshold to electric stimuli varies cyclically when the stimulating electrode or electrodes are moved along a fiber (Kubo and Ono, '34; Hodler, Stämpfli and Tasaki, '52; Rushton, '52). Again, the amplitude of the first phase of diphasic records taken with a fixed short interelectrode distance varies with the position of the recording region with respect to the nodes (Huxley and Stämpfli, '49; the present observations); and the rate of development of this first phase also varies systematically at different regions in an internodal stretch (Huxley and Stämpfli, '49; Hodler, Stämpfli and Tasaki, '52; the present observations). And finally, the membrane current during activity is greater at the nodes than in the internodal regions (Huxley and Stämpfli, '49; Tasaki and Tasaki, '50; the present observations).

But the fact that the properties of myelinated axons differ at the nodes from those encountered in the internodal regions does not prove that propagation is saltatory, if the term is used to imply that the only active segments are the nodal segments. If the myelin sheath is a reasonably good insulator all the differences cited above would appear even though the nodal stretches became active and the propagation of impulses were continuous. In other words, the periodic spatial differences in question may be considered evidence for the insulating properties of myelin, and not for the saltatory conduction of impulses.

That saltatory propagation is possible, that is, that the margin of safety in axons is sufficient for discontinuous activation, has been proved by the observations of Tasaki ('39),

of Huxley and Stämpfli ('49) and of Frankenhaeuser and Schneider ('51), in which an impulse went beyond a blocked region when an adequate external conductor joined the regions behind the block to those ahead of it. But the possibility of discontinuous propagation does not prove that such a propagation occurs normally; it is a necessary, but not a sufficient condition with regard to the theory.

5. *The continuous propagation of impulses in normal myelinated fibers.* As pointed out by Wolfgarm and van Harrevelt ('52), the fact that an impulse may propagate through an internodal segment even when the external impedance connecting the nodes is very high, cannot be regarded as conclusive proof for continuous propagation until more accurate data shall become available on the amount of current necessary for nodal stimulation.

Huxley and Stämpfli ('49) have reported that, while current first leaves and then enters the axon at the nodes, in the internodal segments there is only an outward current flow. They consider this fact as conclusive proof that the internodal segments do not become active, but serve merely as internal conductors for nodal successive activation. The argument is not as decisive as it appears at first sight, because if propagation were continuous inside the myelin sheath, and this sheath had rectifying properties, only an outward flow of current would be observed in the internodal regions.

The present study, however, shows that the membrane current alternates at the internodal segments when impulses propagate, much as it alternates at the nodes. First, we may mention the diphasic character of the direct measurements of membrane current, carried out by differentiating the longitudinal currents with respect to distance, as described in section E. The magnitude of these membrane currents is smaller at the internodal than at the nodal regions but an initial outward flow is always followed by an inward flow (fig. 5).

The diphasic character of the membrane current in the internodal segments is also proved by the changes in the

diphasic records taken with variable interelectrode distances. Let us consider the implications of the theory of saltatory propagation with regard to these changes. In figure 6 A is shown the temporal development of the monophasic spike potential in three successive nodes, a , b and c . Let us first assume that myelin is a perfect insulator, i.e., that it does not allow any outflow or inflow of current. In this case current flows from b to a during the period t_0 – t_1 . A diphasic record taken within the internodal segment a – b would show negativity of the proximal electrode throughout this period. At time t_1 the current would suddenly reverse throughout the internode. If then, the record from electrodes α and β (fig. 6 C) were compared with that from α and γ the first phase of the latter would be greater in amplitude than that of the former, but their duration would be the same. It is clear that if the distal electrode is moved to position δ the duration of the first phase would suddenly increase from t_0 – t_1 to t_0 – t_2 , and that this duration would be the same for positions δ and ϵ . The diphasic records with gradually increasing interelectrode distance, therefore, would behave in the case considered as shown in figure 6 B.

We next assume that myelin is not a perfect insulator, but allows the outward flow of current from the passive internodal segments, as was suggested by Huxley and Stämpfli ('49). At time t_1 current flows from the middle regions of the internode, e.g., from the region in contact with β , both toward a and toward b , i.e., in the segment α – β the current is to the left, and in the segment β – γ it flows to the right. But in those circumstances the record from α to β would still show negativity of α , whereas that from α to γ would already show isopotentiality. In other words, this assumption implies that within an internode the first phase of the diphasic records would last longer for shorter than for longer interelectrode distances.

In fact, as shown in figure 4 the first phase of the diphasic records increases not only in amplitude, but also in duration, when the distal electrode is moved away from the proximal

lead even by steps as small as 0.2 mm, that is, by steps smaller than the internodal distances of the fibers studied. This fact contradicts the hypotheses considered above, and proves that there is not only an outward, but also an inward membrane current in the internodal segments. Let us return to the diagram in figure 6 C and designate the monophasic spikes that develop at the different electrodes by the corresponding Greek letters. The records in figures 1 and 4 indicate that the spike begins earlier at β than at γ . They also indicate that the α spike intersects first the β and later the γ spikes. In addition the β spike also intersects the γ response, since the two electrodes β and γ also yield a typical diphasic record. It is thus clear that throughout the period between the two intersections, α - β and β - γ , β is electronegative both with respect to α and to γ . There is thus an external current flow toward β coming both from the α and from the γ regions. These currents from opposite directions can only coexist if they enter the internodal segment at β .

Summarizing, we find that the axon membrane at the internodal spaces has a demarcation potential (Tasaki, '52); that it develops typical action potentials (Tasaki, '52); that it may be stimulated electrically to originate propagated nerve impulses (Schneider, '50; Tasaki, '50, '52); and that it not only allows outflow, but also receives inflow of current during the passage of nerve impulses (the present study). There is thus no reason to believe that this membrane differs from that at the nodes, nor is there any reason to assume that propagation is saltatory in myelinated fibers.

SUMMARY

The responses of single myelinated fibers in the fine cutaneous branches to the thigh of the 7th spinal nerve of the frog were recorded at different points along the internodal segments.

The latency of the monophasic responses increases gradually with gradually increasing conduction distance (fig. 1). The amplitude of these responses remains practically con-

stant (figs. 2 and 3). The amplitude of the diphasic records taken with a small constant interelectrode distance, on the other hand, shows systematic changes that recur periodically (figs. 2 and 3).

The amplitude and duration of diphasic records taken with progressively larger interelectrode distances increase throughout the internodal stretches (fig. 4). The membrane current alternates not only at the nodes, but also in the internodal myelin-covered regions (fig. 5).

It is shown in the discussion that these features of the responses are incompatible with the theory of saltatory propagation, and that propagation, therefore, is continuous in these, as in other fibers.

LITERATURE CITED

- BLAIR, E. A., AND J. ERLANGER 1933 A comparison of the characteristics of axons through their individual electrical responses. *Am. J. Physiol.*, *106*: 524-564.
- 1936 On the process of excitation by brief shocks in axons. *Am. J. Physiol.*, *114*: 309-316.
- ERLANGER, J., AND E. A. BLAIR 1934 Manifestations of segmentation in myelinated axons. *Am. J. Physiol.*, *110*: 287-311.
- FRANKENHAEUSER, B., AND D. SCHNEIDER 1951 Some electrophysiological observations on isolated single myelinated nerve fibers (saltatory conduction). *J. Physiol.*, *115*: 177-184.
- HODGKIN, A. L. 1951 The ionic basis of electrical activity in nerve and muscle. *Biol. Rev.*, *26*: 339-409.
- HODLER, J., R. STÄMPFLI AND I. TASAKI 1952 Role of potential wave spreading along myelinated nerve fiber in excitation and conduction. *Am. J. Physiol.*, *170*: 375-389.
- 1952 Änderung der Reizschwelle und der Chronaxie längs einer einzelnen, markhaltigen Nervenfasern. *Helv. Physiol. Acta*, *10*: C54-57.
- HUXLEY, A. F., AND R. STÄMPFLI 1949 Evidence for saltatory conduction in peripheral myelinated nerve fibres. *J. Physiol.*, *108*: 315-339.
- KATO, G. 1934 The microphysiology of nerve. Maruzen Co., Ltd., Tokyo.
- KUBO, M., AND S. ONO Cited by G. Kato, 1934.
- KYNASTON THOMAS, P., AND J. Z. YOUNG 1949 Internode lengths in the nerves of fishes. *J. Anat.*, *83*: 336-350.
- LAPORTE, Y. 1951 Continuous conduction of impulses in peripheral myelinated nerve fibers. *J. Gen. Physiol.*, *35*: 323-342.
- LILLIE, R. S. 1925 Factors affecting transmission and recovery in the passive iron nerve model. *J. Gen. Physiol.*, *7*: 473-501.
- LORENTE DE NÓ, R. 1947 Studies from the Rockefeller Institute, Vol. 132.

- LUSSIER, J. J., AND W. A. H. RUSHTON 1952 The excitability of a single fibre in a nerve trunk. *J. Physiol.*, *117*: 87-108.
- ROSENBLUETH, A., W. DAUGHADAY AND D. D. BOND 1943 The electrogram of the ventricle of the turtle's heart. *Am. J. Physiol.*, *139*: 464-476.
- ROSENBLUETH, A., AND J. V. LUCO 1950 The local responses of myelinated mammalian axons. *J. Cell. and Comp. Physiol.*, *36*: 289-332.
- ROSENBLUETH, A., N. WIENER, W. PITTS AND J. GARCÍA RAMOS 1948 An account of the spike potential of axons. *J. Cell. and Comp. Physiol.*, *32*: 275-318.
- SCHNEIDER, D. 1950 Die lokale Reizung und Blockierung im Internodium der isolierten markhaltigen Nervenfasern des Frosches. *Zf. vergl. Physiol.*, *32*: 507-529.
- STÄMPFLI, R. 1952 Bau und Funktion isolierter markhaltiger Nervenfasern. *Ergebn. d. Physiol.*, *47*: 70-165.
- TASAKI, I. 1939 The strength-duration relation of the normal, polarized and narcotized nerve fiber. *Am. J. Physiol.*, *125*: 367-379.
- 1939 The electro-saltatory transmission of the nerve impulse and the effect of narcosis upon the nerve fiber. *Am. J. Physiol.*, *127*: 211-227.
- 1950 Nature of the local excitatory state in the nerve fiber. *Jap. J. Physiol.*, *1*: 75-85.
- 1952 Properties of myelinated fibers in a frog sciatic nerve and in spinal cord as examined with microelectrodes. *Jap. J. Physiol.*, *3*: 73-94.
- TASAKI, N., AND I. TASAKI 1950 Quoted by Stämpfli, 1952.
- WOLFGAM, F. J., AND A. VAN HARREVELD 1952 Modes of conduction in myelinated nerve. *Am. J. Physiol.*, *171*: 140-147.

INTERRELATIONSHIPS BETWEEN CELLULAR RESPIRATION AND CELL GROWTH IN TISSUE CULTURE ¹

BETTY DANES, G. S. CHRISTIANSEN AND P. J. LEINFELDER

*Cellular Physiology Laboratory, Departments of Ophthalmology and Physiology,
College of Medicine, State University of Iowa, Iowa City*

THREE FIGURES

The purpose of this investigation is to demonstrate relationships that exist between cellular respiration and cell growth in tissue culture. In previous studies (Danes and Leinfelder, '51) it has been demonstrated that cellular growth in tissue cultures is altered when respiration is inhibited by hydrogen cyanide. This dependence of certain cells on an aerobic metabolism was shown by the curtailment of cellular migration, the occurrence of aberrant nuclear forms, and cytolysis in cultures exposed to varying concentrations of cyanide. However, in order to relate such cytological changes quantitatively to inhibition of respiration, it was necessary to develop an adequate technique for measuring the respiratory activity of tissue cultures. In this laboratory the Zeuthen ('48) Cartesian diver microbalance for weighing tissue fragments and the Gregg ('47) capillary microrespirometer were adapted into a single apparatus for use in metabolic studies of tissue cultures (Danes, Christiansen and Leinfelder, '53). By use of this apparatus it was possible to weigh, culture, determine the respiration, and finally make morphological studies on the same tissue explant.

¹ This research was made possible through a grant from the Damon-Runyon Memorial Fund.

MATERIALS AND METHODS

By the use of the Cartesian diver microbalance-capillary microrespirometer the respiration of 7-day embryonic chick heart cultures was measured under (1) control conditions and (2) under the influence of various concentrations of hydrogen cyanide (a) during the entire culture period, (b) for the first 24 hours of growth, and (c) after 24 hours of

TABLE 1
Cyanide concentrations

STOCK SOLUTION $\text{Ca}(\text{CN})_2\text{Ca}(\text{OH})_2$	CONCENTRATION OF HCN IN EXPERIMENTAL FLUID
0.650	2.0×10^{-3}
0.313	8.3×10^{-4}
0.081	2.0×10^{-4}
0.0375	8.2×10^{-5}
0.0188	3.8×10^{-5}
0.0090	1.8×10^{-5}
0.00413	7.6×10^{-6}
0.00225	4.0×10^{-6}
0.00125	2.2×10^{-6}
0.00050	8.8×10^{-7}
0.00021	3.8×10^{-7}
0.00015	2.9×10^{-7}
0.00010	1.9×10^{-7}

control growth. These respiratory rate observations were then correlated with observations of cellular activity for each culture.

Respiratory activity was measured initially, and after 12, 24, 36, 48, 60, and 72 hours of growth. Oxygen uptake was calculated in two ways: (1) microliters per milligram reduced weight of the initial explant per hour and (2) microliters per 1000 migrated cells per hour.

$\text{Ca}(\text{CN})_2\text{Ca}(\text{OH})_2$ solution was used as a cyanide source (Robbie, '46). Table 1 lists the concentrations of $\text{Ca}(\text{CN})_2$ -

$\text{Ca}(\text{OH})_2$ used as stock solutions and the resulting molarity of HCN in the experimental media. During cyanide experiments a drop of $\text{Ca}(\text{CN})_2\text{Ca}(\text{OH})_2$ was placed at the base of the capillary respirometer tube instead of potassium hydroxide.

Cytological activity was evaluated by (1) the growth rate expressed as migrated cells per unit time, (2) mitotic index, and (3) the occurrence of aberrant nuclear forms.

OBSERVATIONS

Respiration

Control experiments. Respiration was initially recorded as microliters per milligram reduced weight per hour. Such values showed that the rate of oxygen consumption increased throughout the 72-hour growth period (fig. 1). This fact seemed logically to be related to mitosis and the resulting increased cell population. Since there is no direct relationship between the initial weight of a tissue fragment to be cultured and the subsequent cell mitosis and migration, the measured increased oxygen uptake could not be related quantitatively to cellular activity by such determinations. Therefore, oxygen consumption was calculated as microliters per 1000 migrated cells ($\frac{\mu\text{l O}_2 \text{ total} - \mu\text{l O}_2 \text{ initial}}{\text{number of migrated cells}}$). Such values have greater significance because the increased respiration can be related directly with the increased cellular activity.

As shown in figure 2, the oxygen uptake per cell per hour is higher during the first 12 hours of the culture period than during the subsequent 60 hours.² During the succeeding 60 hours of growth the respiration per cell remains constant and there is remarkably little variation in the oxygen uptake from culture to culture in a given period. The average oxygen uptake expressed as microliters per 1000 migrated cells per hour is 1.76.

² This could be due to cell division within the explant resulting in an increased number of cells which are not observable.

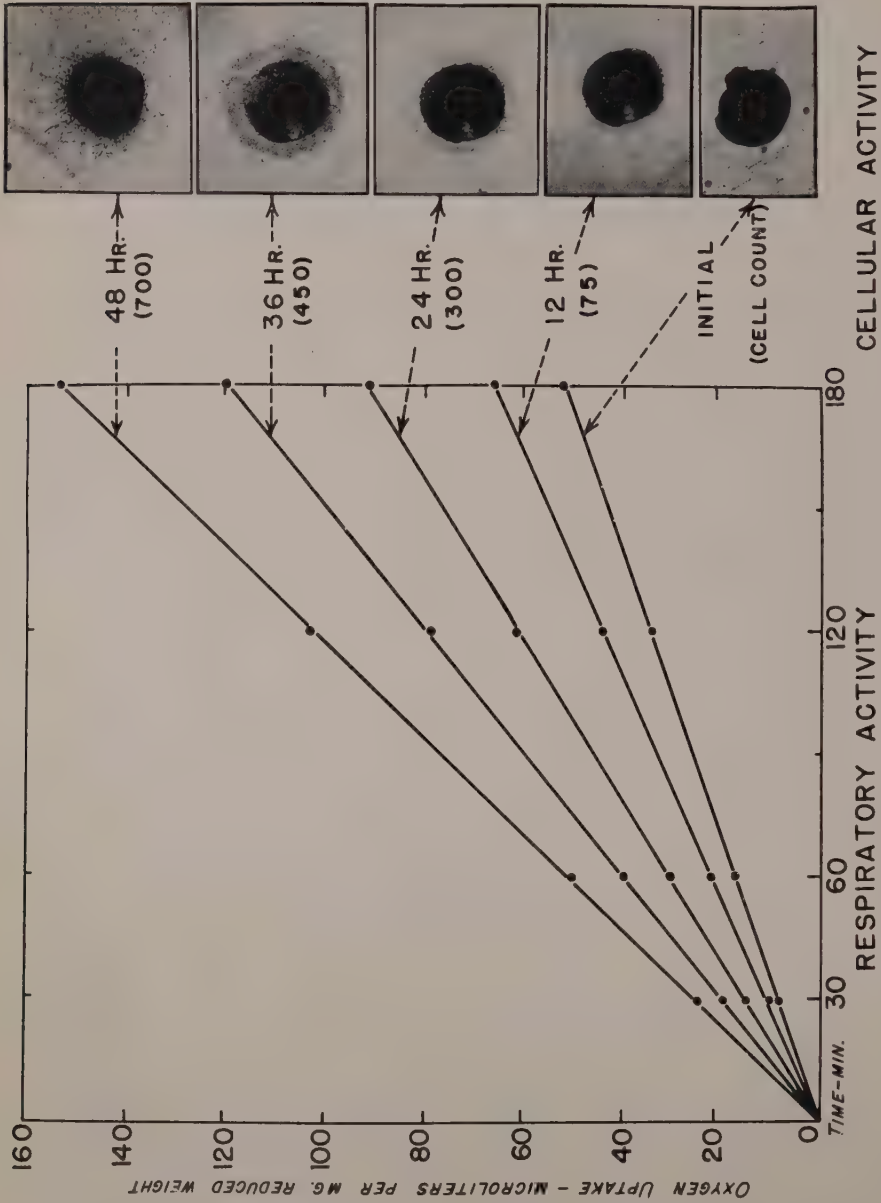


Fig. 1. Correlation of respiration and cellular activity of cultures for 48 hours. Respiration calculated on the basis of the RW of the original cell count.

Hydrogen cyanide inhibition experiments.

Constant exposure. The effects of various concentrations of cyanide on the respiration of cultures exposed constantly for the entire 72 hours of the culture period are shown in table 2. There appear to be two definite phases of inhibition of respiration: (1) With the low concentrations, the first phase of inhibition consists of an abrupt curtailment of res-

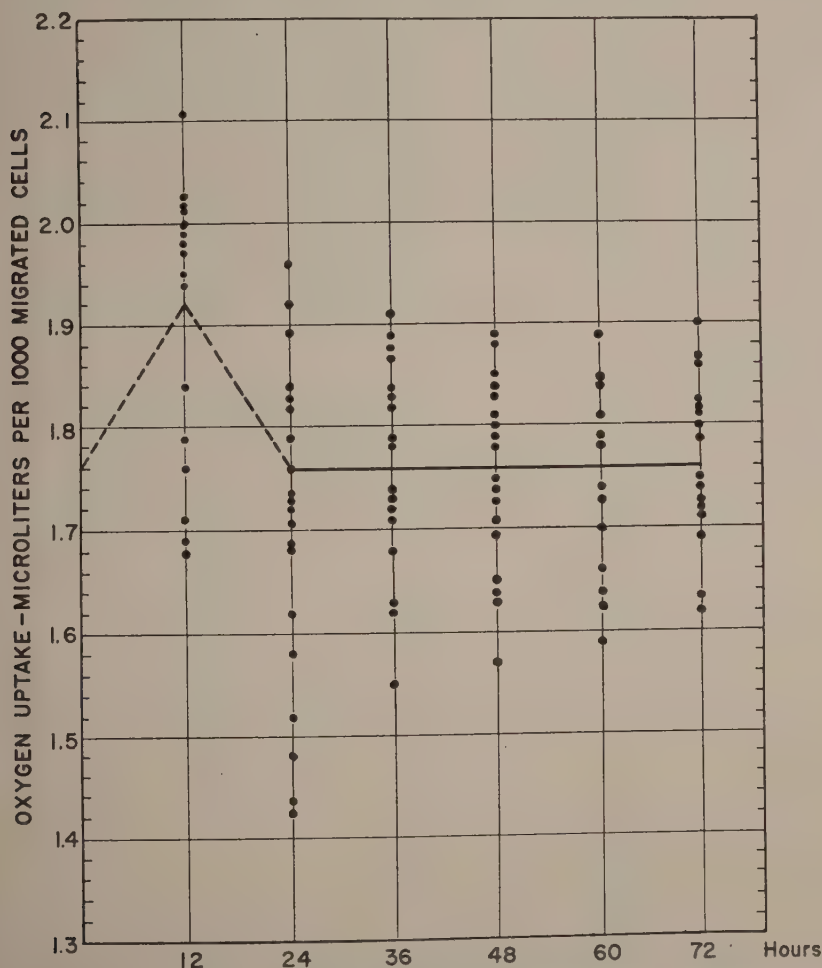


Fig. 2 Respiration of cultures during 72-hour culture period. Respiration calculated on 1000-cell per hour basis.

TABLE 2
Summary table — Respiration and cellular activity of heart cultures exposed constantly to cyanide for 72-hour culture period

CONDITION	NUMBER OF DETER- MINATIONS	Q _{O₂} PER 1000 MIGRATED CELLS						GROWTH (Number of migrated cells)						CELL STRUCTURES 24 HOURS	
														Bilobed nuclei	Mitotic coefficient
		12	24	36	48	60	72	12	24	36	48	60	72		
<i>hours</i>															
Control	29	1.92	1.76	1.76	1.76	1.76	1.76	75	300	450	700	850	1020	1	8.5
Cyanide moles/liter															
1.9 × 10 ⁻⁷	3	2.00	1.73	1.72	1.72	1.72	1.76	78	310	440	695	847	1008	1	9.1
2.9 × 10 ⁻⁷	4	2.22	1.68	1.72	1.72	1.72	1.75	81	292	451	694	868	1018	1	8.1
3.8 × 10 ⁻⁷	4	1.94	1.69	1.75	1.75	1.75	1.74	80	286	420	682	852	1015	1	8.2
8.8 × 10 ⁻⁷	4	1.75	1.56	1.65	1.64	1.64	1.68	83	291	416	672	843	1007	1	6.0
2.2 × 10 ⁻⁶	4	.96	.83	.80	.70	.56	.52	54	203	272	448	507	584	1	6.4
4.0 × 10 ⁻⁶	4	.92	.83	.69	.65	.51	.51	53	201	268	424	501	575	1	5.2
7.6 × 10 ⁻⁶	4	.90	.82	.72	.64	.53	.50	51	203	265	420	489	559	2	3.0
1.8 × 10 ⁻⁵	6	.86	.80	.72	.43	.35	.40	50	200	267	269	297	302	11	..
3.8 × 10 ⁻⁵	5	.66	.63	.44	.21	.05	.05	29	83	92	94	101	50	19	..
8.2 × 10 ⁻⁵	5	.55	.53	.41	..	..	..	20	60	83	0	0	0	72	..
2.0 × 10 ⁻⁴	3	.49	.48	.39	..	..	..	17	33	48	0	0	0	80	..
8.3 × 10 ⁻⁴	6	.24	.12	..	..	..	..	2	3	0	0	0	0	0	..
2.0 × 10 ⁻³	3	..	..	..	..	..	..	0	0	0	0	0	0	0	..

piration to less than one-half the control oxygen uptake. (2) With a concentration of 3.8×10^{-5} M and stronger, there is an increasingly more pronounced inhibition of oxygen uptake until with 10^{-3} M cyanide, oxygen consumption is reduced by approximately 95%. At this concentration significant measurement of respiratory activity cannot be accurately determined because cellular migration is completely curtailed. A residual 5% oxygen uptake remains with increased cyanide concentrations.

Exposure for the first 24 hours of growth. Cultures inhibited during the first 24 hours of growth show some recovery during the subsequent control period (table 3). However, after the removal of the source of cyanide any recovery of respiratory activity is only partial and inversely proportional in amount to the concentration of cyanide. When oxygen uptake has been inhibited more than 90%, measurable evidence of any recovery is not obtained.

Exposure after 24 hours of control growth. The influence of HCN on established growing cultures is greater than on cultures exposed throughout the entire culture period (table 4). A cyanide insensitive respiration ranging from 0.09 to 0.15 μ l per hour or approximately 5% of the control continues even when histologic examination demonstrates that all of the migrated cells are cytolyzed.

Cell growth

Hydrogen cyanide inhibition experiments.

Growth evaluated on the basis of cellular migration and mitosis is affected by the depression of respiration by hydrogen cyanide.

Constant exposure. During the first 24 hours of growth there is no noticeable curtailment of migration under the influence of the lower cyanide concentrations used, but with increasing concentrations of HCN cellular migration is progressively reduced. Although with a concentration of 10^{-3} M hydrogen cyanide there is no recognizable evidence of migra-

TABLE 3
Summary table — Respiration and cellular activity of heart cultures exposed to various concentrations of cyanide for the first 24 hours of growth

CONDITION	NUMBER OF DETERMINATIONS	Q _{O₂} PER 1000 MIGRATED CELLS						GROWTH (Number of migrated cells)					
		12	24	36	48	60	72	12	24	36	48	60	72
Control	29	1.92	1.76	1.76	1.76	1.76	1.76	75	300	450	700	850	1020
Cyanide moles/liter													
1.9 × 10 ⁻⁷	4	1.75	1.71	1.74	1.75	1.73	1.75	80	320	430	690	845	995
8.8 × 10 ⁻⁷	5	1.61	1.64	1.76	1.75	1.75	1.75	78	322	435	700	855	1015
2.2 × 10 ⁻⁶	3	.96	.83	.88	.98	1.04	1.18	57	199	308	556	857	1020
4.0 × 10 ⁻⁶	3	.92	.83	.85	.85	.99	1.06	54	203	299	469	635	890
7.6 × 10 ⁻⁶	3	.89	.81	.80	.85	.94	1.00	50	200	310	483	629	887
1.8 × 10 ⁻⁵	4	.83	.79	.74	.84	.88	.96	27	79	306	469	603	867
3.8 × 10 ⁻⁵	3	.64	.61	.52	.56	.69	.84	23	59	126	224	340	520
2.0 × 10 ⁻⁴	3	.49	.39	.48	.48	.60	.77	15	32	63	140	229	326
8.3 × 10 ⁻⁴	3	.24	.11	.15	.12	.15	.15	0	3	8	14	36	61
2.0 × 10 ⁻³	3	..	..	..	..	..	..	0	0	0	0	0	0

TABLE 4
Summary table — Respiration and cellular activity of heart cultures exposed to various concentrations of cyanide after 24 hours of control growth

CONDITION	NUMBER OF DETERMINATIONS	Q _{O₂} PER 1000 MIGRATED CELLS			GROWTH (Number of migrated cells)	CYTOLYSIS % MIGRATED CELLS
		12	24	36		
Control	29	1.92	1.76	1.76	450	0
Cyanide moles/liter			hours		hours	
8.8×10^{-7}	3	2.10	1.74	1.61	435	0
2.2×10^{-6}	4	1.97	1.83	.58	398	5
4.0×10^{-6}	3	1.97	1.80	.41	310	25
7.6×10^{-6}	3	2.03	1.77	.31	292	40
1.8×10^{-5}	2	1.79	1.71	.11	299	100
3.8×10^{-5}	3	1.95	1.75	.12	320	100
2.0×10^{-3}	3	1.97	1.68	.09	327	100

tion, this does not per se eliminate the possibility of activity within the explant. Besides being related to the concentration of cyanide, the inhibition of cellular activity is more pronounced after prolonged exposure to HCN (table 2). Cultures show a progressively greater curtailment of cell migration throughout the 72-hour exposure period.

The occurrence of cell division gradually decreases with increased curtailment of migration until mitotic figures are not seen (table 2). With the cessation of normal division, the appearance of such abnormal nuclear forms as bilobed nuclei, binucleated cells and fragmented nuclei increase. When cellular migration is nearly completely curtailed, few nuclear abnormalities are seen, indicating that the few cells that migrate from the explant do not attempt to divide.

Exposure for the first 24 hours of growth. Cultures inhibited partially during the first 24 hours of cultivation recover sufficiently at the end of 48 hours of control growth to show a cell count comparable to the control (table 3). With increasing inhibition of cellular migration, the recovery is less complete. Cultures whose migratory activity was completely inhibited by HCN during the first 24 hours of cultivation show no cellular activity during subsequent culturing under control conditions.

Exposure after 24 hours of control growth. The reversibility of partial inhibition at lower concentrations is not seen in cultures exposed to cyanide after 24 hours of control growth. Growing cells exposed to HCN after 24 hours of control growth undergo cytolysis (table 4) at concentrations that do not affect them when exposed constantly from the time of cultivation.

DISCUSSION

Respiration of embryonic chick heart cultures has been determined by several investigators on the basis of the dry weight of the tissue explant (table 5). If our data is calculated in these terms, the value falls near the mean for the reported Q_{O_2} values. However, the measurement of respira-

tory activity on the basis of initial fresh weight or final dry weight of the tissue fragment is unsatisfactory, for no correlation can be made between respiratory activity and cell growth. Therefore, respiration was calculated on the basis of oxygen uptake per 1000 migrated cells per hour. Changes in respiratory activity can then be correlated with cell migration and division.

TABLE 5

Comparison of respiratory rate determinations on embryonic chick heart cultures

SOURCE OF DATA	AGE OF CULTURE	Q _{O₂}
	<i>days</i>	<i>μl/mg. d. wt./hr.</i>
Warburg and Kubowitz ('27)	3	22.9
	24	12.5
	over 1300	12.0
Meier ('31)	1	19.7
Laser ('32, '33)	1	15.4
	5	12.7
	10	7.8
Lipmann ('32)	1	27.5
	3	22.1
	over 30	13.6
From figure 1	0 (explant)	10.4
	$\frac{1}{2}$	13.2
	1	18.2

In this study it was possible to demonstrate that definite alterations in the activity of heart fibroblasts in culture occur when respiratory metabolism is inhibited. Cellular migration, mitosis, and cell integrity are affected when respiration is inhibited by hydrogen cyanide (fig. 3).

Cellular migration can be unaffected, partially or completely curtailed by inhibition of respiration. With slightly lowered oxygen uptake, there is no evident inhibition of cellular migration. Thus cells may migrate from the explant when respiration is slightly curtailed. Further evidence to substantiate this statement is seen in cultures partially inhibited

for only the first 24 hours and then allowed to grow under control conditions for 48 hours. Cellular migration is soon comparable to that in control cultures, even though the respiration remains slightly depressed. However, when the inhibition of oxygen uptake is increasingly inhibited, migration

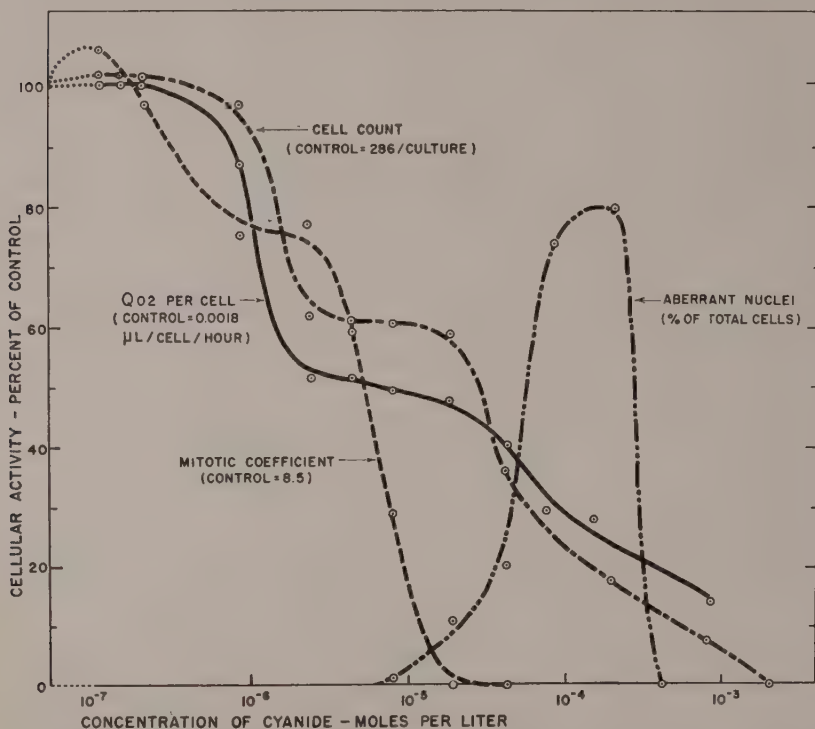


Fig. 3 Summary to show certain relationships between cell growth and respiration. Respiration and cellular activity of 24-hour cultures under constant exposure to HCN.

is progressively curtailed. Complete absence of migration occurs when respiratory activity is nearly totally suppressed. This assuredly indicates that cellular migration is dependent on aerobic metabolism.

Inhibition of respiration also has an effect on mitosis, for as shown in figure 3, the occurrence of mitotic figures rapidly decreases as the oxygen uptake is curtailed by increasing

concentrations of HCN. In addition the appearances of numerous aberrant nuclei in partially inhibited cultures suggests that normal cell division, although being instigated, is not being completed. The absence of abnormal forms when respiration is nearly totally inhibited indicates that the few cells that migrate do not attempt to divide.

Maintenance of the integrity of the cells appears to be dependent on aerobic respiration, for cells whose respiration is nearly totally curtailed from the time of culturing die. However, some depression of respiration is compatible with continuation of cell life. Cells in control cultures show cytolysis when respiration is partially curtailed (table 4). All of this evidence indicates that the embryonic chick heart fibroblast is almost completely dependent upon an aerobic energy system.

Respiratory activity throughout the culture period in both control and inhibited cultures indicated that mitosis may occur in the explant, following which one of the resulting daughter cells migrates into the clot.

CONCLUSIONS

1. The respiration of chick heart fibroblast is remarkably constant over a period of 72 hours. The average oxygen uptake expressed as microliters per 1000 migrated cells per hour is 1.76.

2. Oxygen uptake of the growing fibroblasts is reduced by increasing concentrations of cyanide. At all concentrations of cyanide used, the respiration rate, although lower than the control, remains constant throughout the culture period.

3. Definite alterations in the activity of heart fibroblasts occur when respiration is inhibited. The curtailment of cellular migration, the cessation of normal division and the occurrence of aberrant nuclear forms, and cytolysis indicate that cell growth is affected by inhibition of cellular respiration.

LITERATURE CITED

- DANES, B., AND P. J. LEINFELDER 1951 Cytological and respiratory effects of cyanide on tissue cultures. *J. Cell. and Comp. Physiol.*, *37*: 472.
- GREGG, J. R. 1947 Microrespirometer. *Rev. Scient. Instr.*, *18*: 514.
- LASER, H. 1932 Eine Methode zur manometrischen Messung des Stoffwechsels von Gewebekulturen während des Wachstums. *Biochem. Ztschr.*, *251*: 2.
- 1933 Flächengrösse und Wachstum von Gewebekulturen. *Ztschr. f. Krebsforsch.*, *39*: 384.
- LIPMANN, F. 1932 Versuche zur Methodik der Messung des Zuwachses "in vitro" wachsender Gewebe durch messung des Vmsatzanstiegs. *Biochem. Ztschr.*, *244*: 177.
- MEIER, R. 1931 Zur Methodik der Stoffwechseluntersuchungen an Gewebekulturen. I. Atmungsmessung an Gewebekulturen. *Biochem. Ztschr.*, *231*: 247.
- 1931 Zur Methodik der Stoffwechseluntersuchungen an Gewebekulturen. II. Gewichtsbestimmung an einzelnen Gewebekulturen. Gewichtszunahme und Flächenzunahme. *Biochem. Ztschr.*, *231*: 253.
- ROBBIE, W. S. 1946 The quantitative control of cyanide in manometric experimentation. *J. Cell. and Comp. Physiol.*, *27*: 181.
- WARBURG, O., AND F. KUBOWITZ 1927 Stoffwechsel wachsender Zellen (Fibroblasten, Herz, Chorion). *Biochem. Ztschr.*, *189*: 242.
- ZEUTHEN, E. 1948 A Cartesian diver balance weighing reduced weights (R.W.) with an accuracy of $\pm 0.001 \gamma$. *Compt. rend. Lab. Carlsberg, Ser. Chim.*, *26*: 243.

ENZYME DISTRIBUTION IN ISOLATED PARTICULATES OF RAT PERIPHERAL NERVE¹

L. G. ABOOD AND R. W. GERARD

*Division of Psychiatry and Department of Biochemistry, University of Illinois
College of Medicine, Chicago*

TWO FIGURES

In the cell bodies of neurones and glia there is a marked localization of energy-yielding oxidizing and phosphate-splitting enzyme systems in the cytoplasmic mitochondria. The less important glycolytic system appears in the soluble fraction and choline esterase is divided, but largely in the particulates (Abood and Gerard, '52a). If either ATP or ACh is primarily concerned with the actual functioning machinery, rather than serving as an energy source (Abood, Gerard and Tschirgi, '51b; Gerard and Taylor, '53; Falk and Gerard, '54), its enzymes would be expected mainly in the membrane — where, indeed, they have been reported for the squid giant axone (Libet, '48; Nachmansohn et al., '43). For the nerve fiber, especially, the energy of the resting and active metabolism appears to be concerned mainly with maintenance of the membrane and of the ion populations it separates; so oxidizing systems might also be expected in or near the membrane. It seemed, therefore, of great interest to learn if the enzymes controlling oxidation and phosphate metabolism (and ACh) are localized in mitochondria in peripheral nerve. The finding (Gasser, '52) that these particulates are clustered at the nodes of Ranvier, where the "naked" membrane presumably does the bulk of ionic work, is of especial interest in this connection; and the very rich mitochondrial content of Schwann cells is also significant. This

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study is concerned with the localization of enzymes in particulates of peripheral nerve, using separation techniques. Functional inferences can be drawn from their distribution.

MATERIALS AND METHODS

Male Sprague-Dawley rats weighing about 350 gm and fed Purina Dog Chow *ad libitum* were used throughout. Nerves were removed at room temperature, after stunning the animal by a blow on the head; for preliminary analyses showed that chilling the rats in acetone-dry ice immediately after stunning did not alter the amount and distribution of phosphorylated intermediates. Sciatic, brachial, and spinal nerves were combined and, after removal of connective tissue, homogenized for 5 minutes (Potter-Elvehjem homogenizers); in ice-cold isotonic KCl for direct enzyme studies, in 0.25 M sucrose for particulate separation.

Phosphorylated intermediates were analyzed following LePage and Umbreit ('49); the acid insoluble phosphorus components, following Schneider ('45); succinic dehydrogenase and cytochrome oxidase, following Schneider and Potter ('43); malic dehydrogenase and DPN-cytochrome *c* reductase, following Potter ('49); adenylypyrophosphatase, following DuBois and Potter ('43), but with 0.05 M histidine buffer replacing barbital buffer; alkaline phosphatase, following Bodansky ('37); aldolase, following Sibley and Lehninger ('49); adenylic kinase, following Kornberg ('50); and adenylic deaminase as follows. To conical centrifuge tubes were added: 0.25 cm³ of 0.1 M histidine buffer, pH 7.5, 0.2 cm³ of 10⁻³ M AA, and 0.2 cm³ of tissue. The mixture was incubated at 37° for 20 minutes and the reaction then stopped with 1.0 cm³ of 3% perchloric acid; 0.2 cm³ was removed. The optical density at 260 mμ was determined on a 0.2 cm³ sample with the Beckman DU Spectrophotometer.

The cytoplasmic particulates were prepared by a modification of the method of Schneider ('48). Centrifuging was done in a Spinco ultracentrifuge at 0° C. Fragments, stroma, and Schwann nuclei were removed by three successive cen-

trifugations at $1000 \times g$ for 5 minutes, fraction R_1 ; mitochondria, a few fragments of Schwann nuclei and small axones (axoplasm extruded), and some "microsomes" were next spun down at $10,000 \times g$ for 20 minutes, fraction R_2 ; "microsomes," spherical particles ranging in diameter from 100–500 $m\mu$ were then sedimented at $20,000 \times g$ for 30 minutes, fraction R_3 ; and finally particles less than 100 $m\mu$ in diameter were obtained at 60,000 to 70,000 $\times g$ for 30 minutes, fraction R_4 . The final supernatant, fraction S, was almost clear; lipid agglutinations, which commonly formed, could be removed by straining through cheesecloth.

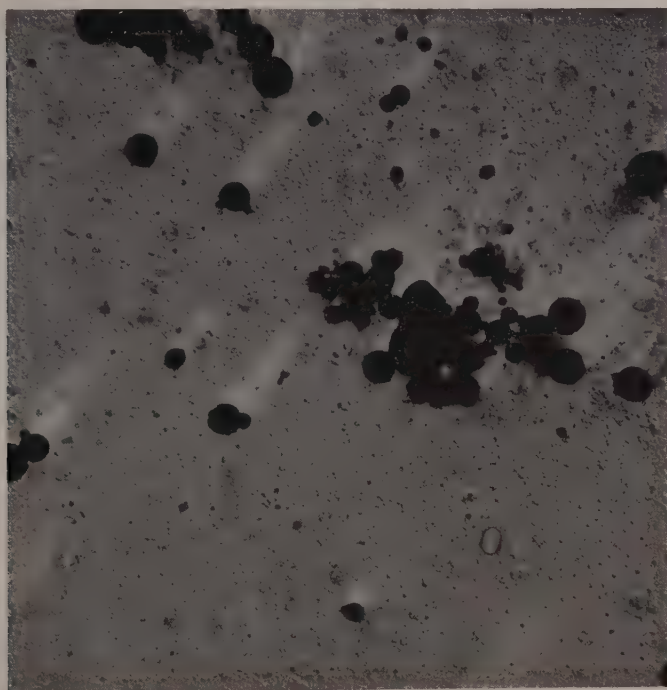


Fig. 1 Electron micrograph of isolated mitochondria (R_2) from rat peripheral nerve. A drop of the mitochondrial suspension (0.25 M sucrose) was placed on screen and exposed to osmic acid vapors for 12 hours. Sucrose was washed thoroughly with distilled H_2O , the preparation was then dried and "shadow-cast." The instrument was an RCA-EMU. Magnification $\times 5,000$.

The various fractions were examined under ordinary or phase contrast microscopes, unstained or after staining with Janus green B or methylene blue; and photomicrographs made under the electron microscope, usually after shadow casting.



Fig. 2 Electron micrograph of "microsomes" (R_4) from peripheral nerve. Magnification $\times 8,000$.

RESULTS

Differential centrifugation achieved a fairly complete separation of the different sized particles, the separate fractions being predominantly Schwann nuclei and nerve fragments (R_1), mitochondria (R_2), and microsomes (R_3 and R_4), as mentioned above. The nuclei stained well with both dyes, which set them off from the few unhomogenized nerve fragments and myelin figures also present. The mitochondria, with very few fragments of small nerve fibers, connective

tissue, nuclei, and myelin figures, were seen well with phase contrast and varied in shape from rods to spheres and in diameter from 0.5 to 1.5 μ . They stained with Janus green B much more quickly and deeply than did the Schwann nuclei and stained somewhat with methylene blue. The microsomes were seen as good spheres under the electron microscope¹;

TABLE 1

Phosphorylated intermediates of rat sciatic and spinal nerves

Average of 4 experiments agreeing within 7%

	SCIATIO	SPINAL	RATIO
	$\mu M/gm$	$\mu M/gm$	
Total P	12.2	30.6	2.5
Inorganic P	5.2	15.8	3.0
Glucose-1-Phosphate	0.15	0.20	1.3
Glucose-6-Phosphate	1.00	1.35	1.4
Fructose Phosphates	0.31	0.27	0.9
Phosphoglyceric Acid	0.60	0.82	1.4
ATP	0.76	0.68	0.9
ADP	0.20	0.37	1.8
AA	0.30	0.50	1.7
Phosphocreatine	2.00	5.42	2.7

TABLE 2

Nitrogen and acid insoluble phosphorus distribution of rat nerve particulates fractions

	TOTAL	% OF TOTAL ACTIVITY					% RECOVERY
		R ₁	R ₂	R ₃	R ₄	S	
	$\mu M/gm$						
Nitrogen	933	22.5	18.4	13.5	22.6	24.4	101.4
Phospholipid P	90.0	17.6	18.0	17.8	42.5	1.1	97.0
Nucleic Acid P	2.96	5.2	10.4	17.0	63.2	7.0	102.8
Phosphoprotein P	1.26	41.0	40.0	4.0	3.3	6.6	94.9

¹ The authors are indebted to Miss Elizabeth Bush for the electron micrographs.

TABLE 3
Intracytoplasmic distribution of adenylate enzymes and aldolase in rat nerve

ENZYME	SUBSTRATE	ACTIVITY OF WHOLE HOMOGENATE	% OF TOTAL ACTIVITY				S	% RECOVERY
			R ₁	R ₂	R ₃	R ₄		
Adenylpyrophosphatase-3 (Ca-activated)	ATP	5.0 γ P/ mg/15'	16	35	10	30	2	93
Adenylpyrophosphatase-3 (Mg-activated)	ATP	6.8 γ P/ mg/15'	10	20	6	55	3	94
Adenylpyrophosphatase-2	ADP	5.5 γ P/ mg/15'	8	20	5	54	4	91
Adenylpyrophosphatase-1	AA	0.7 γ P/ mg/15'	20	23	9	40	2	94
Adenylic Deaminase	AA	1.1 γ AA/ mg/15'	0	20	8	70	0	98
Adenylic Kinase	ADP	15 γ ATP/ mg/15'	0	20	3	65	5	93
Alkaline Phosphatase	Sodium β -glycero- phosphate	0.5 γ P/ mg/30'	30	50	10	5	3	98
Aldolase	HDP	24 μ M HDP/ hr./gm	0	2	0	1	90	93

ranging in size between 100 m μ in fraction R₃, 50 and 100 in fraction R₄ and below 50 in fraction S (with an occasional larger sphere still present) (figs. 1 and 2).

The analytic findings are presented in 6 tables, which require but few text comments. The total phosphorylated intermediate content (table 1) is 2.5 times greater in spinal than in sciatic nerves. The ratios for inorganic P and creatin

TABLE 4

Distribution of oxidases in cytoplasmic fractions of rat peripheral nerves

OXIDASE	QO ₂ ¹	% OF TOTAL ACTIVITY ²					% RECOVERY
	Whole nerve	R ₁	R ₂	R ₃	R ₄	S	
Succinic dehydrogenase	2.3	6	61	25	2	0	94
Malic dehydrogenase	1.6	4	20	1	2	65	92
DPN-Cytochrome C reductase	2.2	5	58	4	1	30	98
Cytochrome oxidase	11.0	5	60	20	3	4	92

¹ QO₂ = Microliters oxygen/mg fat free dry weight/hour.

² Average of 4 experiments, agreeing within 15%.

TABLE 5

P/O Ratios for nerve homogenates

	NO. EXPERIMENTS	MICROATOMS O ₂ UTILIZED	MICROMOLES P ESTERIFIED	P/O
Pyruvate	3	0.67	1.04	1.6
Malate	3	0.58	0.81	1.4
Succinate	4	0.87	1.22	1.4
Pyruvate and malate	4	0.67	1.48	2.2
Glucose	4	0.25	0.38	1.5

Each flask contained the following: 8×10^{-3} M MgCl₂, 8×10^{-3} M KF, 10^{-3} M K-ATP, 5×10^{-4} M DPN, 10^{-3} M cytochrome C. Fifteen micromoles K-PO₄, pH 7.5, 5×10^{-3} M glucose, 1 mg yeast hexokinase, mitochondria from 200 mg of nerve, KOH in center well. Incubation time 20 minutes, T = 37°.

P are close to three, those for most of the carbohydrate (G-1-P, G-6-P, phosphoglyceric acid) and adenylic (ADP, AA) phosphates from 1.3 to 1.5, but ratios for fructose P and ATP actually one or less. Perhaps only the three-fold differences are significant; their relation to histology and energy release will be discussed. These soluble substances appear largely in the S fraction.

TABLE 6
Turnover of phosphorylated intermediates of whole nerve during oxidation of glucose

	UNITS	INORGANIC P	ATP AND ADP P	GLUCOSE-6-P
After incubation	$\mu\text{M/gm}$	8.20	0.25	1.80
Specific activity of P fractions	counts/min./ $\mu\text{g/P}$	650	238	213
	PHOSPHO- GLYCERIC ACID	PHOSPHO- LIPID P	PHOSPHO- PROTEIN P	$\mu\text{L O}_2/30'$
After incubation	2.5	91.5	2.7	9.0
Specific activity of P fractions	136	0.76	13.4	

Warburg flasks contained following: 3 cm³ of phosphate-free Krebs-Ringer's solution, 0.05 M glycylglycine buffer, 0.05 M glucose, 10 μe of P³², 200 mg rat sciatic and spinal nerves. Incubation time 30 min., gas phase was air. T = 37°.

Nitrogen is distributed (table 2) roughly alike in the 5 fractions, R₃ being low; and phospholipid P is similar except that R₄ contains double the average and S, only a trace. Nucleic acid P is overwhelmingly in R₄; phosphoprotein P, mostly in R₁ and R₂.

The enzymes of the adenylic system and other phosphatases (table 3) are present in the supernatant only as traces. They are mostly found in the R₄ fraction (between 40 and 55% for the three adenylypyrophosphatases, 70% for adenylic

deaminase and kinase) but the Ca-activated ATP-ase and the scant alkaline phosphatase are richest in R_2 (35 and 50% respectively, and remainder in R_1 and R_4).

By contrast (table 4), aldolase is almost entirely in S, malic dehydrogenase 65% there, and the other oxidizing components (succinic dehydrogenase, DPN-cytochrome *c* reductase and, 5-fold richest, cytochrome oxidase) 60% in R_2 . Only the reductase is significantly present in S (30%), and none appears in R_4 .

Oxidative phosphorylation (table 5) is about 1.5 P/O for pyruvate, malate, or succinate; 2.2 for pyruvate plus malate at the same O_2 consumption level. Glucose is oxidized only two-fifths as fast as the other substrates and the P/O ratio is only 0.6. During glucose oxidation (30 min. incubation), ATP and ADP decrease considerably while G-6-P and P glyceric acid increase (table 6 compared to table 1). With P^{32} present, the specific activities (table 6) of the above rose to about one-third that of the inorganic P (P glyceric acid only to 20%), while phosphoprotein rose to 2% and phospholipid to 0.2%.

DISCUSSION

The role of mitochondria and other particulates in the affairs of nerve fibers has had some attention from histologists. The number and distribution of these particles, their changes with physiological state and, recently, evidence as to their composition permit some inferences regarding function. In general, the location and composition of mitochondria in the nerve fiber resemble the situation in neurone and glial cell bodies (Abood et al., '52a) and in other cells.

The number of mitochondria in perikarya, per unit volume of cytoplasm, is distinctive for each of the main cranial nerve nuclei (Thurlow, '17). In spinal ganglion cells, these elements are altered by thiamin deficiency (Ma, '25) but not by avulsion of the axon (Marinesco and Tupa, '22). They are more numerous during regeneration (Nageotte, '22) and, in general, a rise or fall in number has been related to rise or fall of activity level (Cowdry, '22).

In peripheral nerve, the mitochondria do seem to be located where metabolism is high or energy needed. Most are in the Schwann cells, around the nuclei, which are unusually rich (Nageotte, '32; Cowdry, '14); but they are also present in axoplasm (Rosza et al., '50) and are concentrated at synaptic terminals (Bodian, '51) and near nodes (Gasser, '52). The need of metabolic energy to maintain membrane polarization (Gerard, '46; Höber, '46) to keep potassium in (Harris, '41; Turner et al., '51) and sodium out, and to form or remove particular substances — quaternary ions (Lorente de Nó, '49; Geiger and Magnes, '53), including ACh (Nachmansohn et al., '43), ATP and CrP (Gerard and Tupikova, '39), etc. — is too well known to need elaboration. (See Abood et al., '52c, for review.) The heavy concentration of oxidizing enzymes in the mitochondria and the location of mitochondria in relation to (but not actually in) active surfaces is thus understandable. The high content of "phosphoprotein" in mitochondria is noteworthy.

Dephosphorylating enzymes and adenylate kinase, as well as phospholipids and nucleic acids, are associated mainly with the small microsome fraction (R_4 , 50–100 m μ spheres). Only the Ca-activated ATP-ase, in contrast to the Mg-activated one, and the feeble alkaline phosphatase are relatively rich in the mitochondria. The location of these microsomes would, thus, contribute important evidence as to the role of phosphates in nerve function. If the axon proper is free of lipid, for which there is evidence (Engström and Lüthy, '50), the lipid-rich microsomes could not be in the axoplasm nor have such elements been observed in the axoplasm (Rosza et al., '50). Further, so far as structural comparisons are meaningful between rat nerve fibers and the squid giant axon, ATP-ase is in the membrane or surrounding structures (Libet, '48). Dr. Libet kindly sent us two samples of separated giant fiber sheath and axoplasm; and we found, in preliminary tests, that the small spheres were present in the sheath fraction and contained most of the ATP-ase. Whether these microsomes are present as such in the sheath, perhaps as a

mosaic, or are separated from larger structures is uncertain. In rat nerve they are related to myelin (Sjöstrand, '50), and may even be in the Schwann cells.

Some evidence is available on the path and significance of energy-rich phosphate turnover in nerve. The Mg-activated ATP-ase, adenylypyrophosphatase 1, and the ADP-ase, adenylypyrophosphatase 2, are present with nearly identical overall activity and with similar distribution in the fractions, mainly in R_4 . Since R_4 also contains sufficient adenylate kinase to convert ADP to ATP and AA as fast as ADP and ATP are dephosphorylated, it may be that ADP is split entirely via dismutation by the kinase and dephosphorylation of the ATP so produced. This has been shown for liver fractions (Novikoff et al., '52). Adenylypyrophosphatase 2 would then be identical with the Mg-activated ATP-ase.

Peripheral nerve contains about as high a concentration of energy-rich phosphates and related intermediates as does cortex, despite a 20- to 30-fold difference in oxidizing activity and energy release. Oxidative phosphorylation does occur in nerve, but at a lower P/O ratio than in the C.N.S. The overall decrease in $\sim P$ which occurs during activity represents a decreased rate of formation or increased rate of splitting or both. A decreased formation has been demonstrated with electrical stimulation of brain mitochondria (Abood and Gerard, '53) and with constant current contracture of muscle (Taylor and Abood, '53), and other evidence suggests an increase of responsiveness of brain (Abood et al., '52d) and of muscle (Ling and Gerard, '49; Jenerick and Gerard, '53) with lowered $\sim P$. If a decreased rather than an increased turnover is found to be associated with activity, it would prove a role of phosphates in the machinery of conduction rather than as an energy source. Since normal turnover maintains a high K/Na, a fall would favor the entry of Na^+ and loss of K^+ . A role of $\sim P$ in conduction would also fix the presumptive location of the ATP-splitting enzymes in microsomes within the membrane.

SUMMARY

The content of phosphorus compounds and enzymes and the rate of turnover have been determined for rat sciatic and spinal nerves, which are not the same, and the distribution measured of various substances and enzymes in the particulate fractions. Oxidases are mainly in mitochondria, many phosphate enzymes in small microsomes ($< 0.1 \mu$), and glycolytic enzymes in the supernate. A Ca-activated ATP-ase, mostly in mitochondria, is distinguished from a Mg-activated one, mainly in small microsomes. The small microsomes also contain 40% of the total phospholipid and 60% of the nucleic acid of nerve. The role of particulates in function and metabolism is considered in terms of their number, location, and content. Mitochondria, stations of oxidative energy, are located in the vicinity but probably not the site of physiological action. Small microsomes, perhaps containing part of the functioning machinery (phosphates), seem to be located in the membrane or its sheath. Turnover of soluble phosphate esters is considered in relation to energy supply and to responsiveness of nerve.

LITERATURE CITED

- ABOOD, L. G., AND R. W. GERARD 1952d Phosphorus metabolism and function in the nervous system. *Fed. Proc.*, **11**: 1.
- ABOOD, L. G., R. W. GERARD, J. BANKS AND R. D. TSCHIRGI 1952a Substrate and enzyme distribution in cells and cell fractions of the nervous system. *Am. J. Physiol.*, **168**: 728.
- ABOOD, L. G., R. W. GERARD AND S. OCHS 1952e Electrical stimulation of metabolism of homogenates and particulates. *Am. J. Physiol.*, **171**: 134.
- ABOOD, L. G., R. W. GERARD AND R. D. TSCHIRGI 1952b Spatial and chemical exchange of phosphate in the resting and active nervous tissue. In "Phosphorous Metabolism." Vol. II, ed. by W. D. McElroy and B. Glass. Johns Hopkins Univ., Baltimore, Md.
- BODANSKY, A. 1937 Notes on the determination of serum inorganic phosphate and serum phosphatase activity. *J. Biol. Chem.*, **120**: 167.
- BODIAN, D. 1951 In "Nerve Impulse," Vol. I., Macy, Jr., Foundation, N. Y., p. 108.
- COWDRY, E. V. 1914 The development of the cytoplasmic constituents of the nerve cells of the chick. I. *Am. J. Anat.*, **15**: 389.

- COWDREY, E. V. 1926 The reactions of mitochondria to cellular injury. *Arch. Path. and Lab. Med.*, 1: 237.
- DUBOIS, K. P., AND V. R. POTTER 1943 The assay of animal tissues for respiratory enzymes. III. Adenosintriphosphatase. *J. Biol. Chem.*, 120: 167.
- ENGSTRÖM, A., AND H. LÜTHY 1950 The distribution of mass and lipids in the single nerve fibers. *Exp. Cell. Res.*, 1: 81.
- FALK, G., AND R. W. GERARD 1954 Effect of microinjection of salts and ATP on membrane potential in muscle. *J. Cell. and Comp. Physiol.*, in press.
- GASSER, H. S. 1952 In "The Neuron." Symposia on Quantitative Biology, XVII, p. 34.
- GERARD, R. W. 1946 Nerve metabolism and function. *Ann. of N. Y. Acad. Science*, 47: 575.
- GERARD, R. W., AND R. D. TAYLOR 1953 Muscle and Nerve—Physiologic Orientation. *Am. J. Med.*, 15: 83.
- HARRIS, J. E. 1941 The influence of the metabolism of human erythrocytes on their potassium content. *J. Biol. Chem.*, 141: 579.
- HÖBER, R. 1946 The membrane theory. *Ann. of N. Y. Acad. Science*, 47: 381.
- JENERICK, H. P., AND R. W. GERARD 1953 Membrane potential and threshold single muscle fibers. *J. Cell. and Comp. Physiol.*, 42: 79.
- KORNBERG, A. 1950 Reversible enzymatic synthesis of diphosphopyridine and inorganic pyrophosphate. *J. Biol. Chem.*, 182: 779.
- LEPAGE, G. H. in Umbreit, W. W., R. H. BURRIS AND J. F. STAUFFER 1949 In "Manometric Techniques and Tissue Metabolism." 2nd ed., Burgess Publishing Co., Minneapolis, Minn.
- LIBET, B. 1948 ATP-ase in nerve. *Fed. Proc.*, 7: 72.
- LORENTE DE NÓ, R. 1949 On the effect of certain quaternary ammonium ions upon frog nerve. *J. Cell. and Comp. Physiol. Supp.*, 33: 3.
- MA, W. C. 1925 A study of the mitochondrial elements of the spinal ganglion cells of beriberi fowls. *Am. J. Anat.*, 36: 215.
- MARINESCO, G., AND A. TUPA 1922 Recherches histo-pathologiques sur les mitochondries. *Compt. rend. soc. de biol.*, 37: 292.
- NACHMANSOHN, D., AND A. L. MACHADO 1943 The formation of acetylcholine. A new enzyme: "Choline acetylase." *J. Neurophysiol.*, 6: 397.
- NACHMANSOHN, D., H. B. STEINBACK, A. L. MACHADO AND S. SPIEGELMAN 1943 Localization of enzymes in nerves. II. *J. Neurophysiol.*, 6: 203.
- NAGEOTTE, J. A. 1922 L'organisation de la matière dans ses rapports avec la vie. Libraire Felix Alcan.
- 1932 In "Cytology and Cellular Pathology of the Nervous System." Ed. W. Penfield, P. B. Hoeber, N. Y.
- NOVIKOFF, A. B., L. HECHT, E. PODBER AND J. RYAN 1952 Phosphatases of rat liver. I. The dephosphorylation of adenosintriphosphate. *J. Biol. Chem.*, 194: 153.
- POTTER, V. R. 1949 In "Manometric Techniques and Tissue Metabolism," Burgess Publishing Co., Minneapolis, Minn.
- ROSZA, G., C. MORGAN, A. SZENT-GYÖRGYI AND R. W. G. WYCKOFF 1950 The electron microscopy of myelinated nerve. *Biochim. et Biophysica Acta*, 6: 13.

- SCHNEIDER, W. C. 1945 Phosphorous compounds in animal tissues. I. Extraction and estimation of desoxypentose nucleic acid and of pentose nucleic acid. *J. Biol. Chem.*, 161: 293.
- 1948 Intracellular distribution of enzymes. III. The oxidation of octanoic acid by rat liver fractions. *J. Biol. Chem.*, 176: 259.
- SCHNEIDER, W. C., AND V. R. POTTER 1943 The assay of animal tissue for respiratory enzymes. II. Succinic dehydrogenase and cytochrome oxidase. *J. Biol. Chem.*, 149: 217.
- SIBLEY, J. A., AND A. L. LEHNINGER 1949 Determination of aldolase in animal tissues. *J. Biol. Chem.*, 177: 859.
- SJÖSTRAND, F. 1950 Electron-microscopic demonstrations of membrane structure isolated from nervous tissue. *Nature*, 165: 482.
- TAYLOR, R. D., AND L. G. ABOOD In preparation.
- TEBNER, C., L. V. EGGLESTON AND H. A. KREBS 1951 The role of glutamic acid in the transport of potassium in brain and retina. *Biochem. J.*, 47: 139.
- THURLOW, M. 1917 Quantitative studies on mitochondria in nerve cells. *Embryol.*, Carnegie Institute, Washington, D. C., 6: 35.

EFFECT OF MICRO-INJECTED SALTS AND ATP ON THE MEMBRANE POTENTIAL AND MECHANICAL RESPONSE OF MUSCLE¹

G. FALK² AND R. W. GERARD

*Department of Psychiatry, Neuropsychiatric Institute,
University of Illinois, Chicago*

Varying the external concentration of potassium ions produces alterations in the resting membrane potential of nerve and muscle in approximate agreement with the predictions of the Nernst equation (Curtis and Cole, '42; Ling and Gerard, '50). The membrane theory of Bernstein ('02) has been modified by recent work (Boyle and Conway, '41; Levi and Ussing, '48), which indicates that the membrane is permeable not only to potassium, but also to chloride, both ions being distributed according to their electrochemical potentials. Evidence that labeled sodium is free to exchange in the cell has necessitated the postulation of a special mechanism which extrudes sodium (Ussing, '49). A precise analytical treatment of membrane potentials as a function of ionic concentrations and conductances has been given in the extensive researches of Hodgkin and co-workers ('51; '52a, b).

That metabolism plays an important role in the maintenance of a potential gradient across the cell surface seems evident. It is still not clear whether the role of metabolism is direct, i.e., whether it provides an immediate source of energy for the

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potential, or whether its function is indirect in the sense of supporting the semipermeable properties of the membrane or the concentration of labile ions (as CrP).

The present investigation, using micro-injection as a means of altering intracellular concentrations of salts or metabolic intermediates in single muscle fibers, and observing membrane potential and mechanical changes, has led to some definitive conclusions. Most striking, ATP affects potential and length only when added outside the fiber; injected ATP is ineffective.

METHODS

Resting membrane potentials were recorded from fibers of the isolated sartorius of *Rana pipiens* (Ling and Gerard, '49a) in phosphate-buffered Ringer solution containing 2.5 mM K. The intracellular capillary (0.5–1.0 μ tip diameter) served both for electrical recording and injection. Capillaries were filled without boiling, necessary with heat-labile materials, by snipping out an essentially cylindrical flow-resisting portion between tip and shank and fitting the tip into the new end. With the large end of the shank immersed in the desired fluid, this now rose to the tip by capillarity alone. The joint was finally sealed with Plicene cement (Cenco).

Each needle was calibrated before and after use by applying a constant pressure (up to 3 atmospheres) from a nitrogen tank and measuring the diameter increase with time of a drop-let ejected under oil. Surface tension errors were negligible, since flow remained nearly constant for each needle at a given pressure, despite changing diameter. The amount of fluid injected, 10^{-8} to 10^{-6} cm³ (equal to a cube of side 20 to 100 μ) corresponded to 0.004 to 0.4% of the fiber volume. Although the solutions injected were highly concentrated (generally 3 M), the amount of solute was small, so that swelling and osmotic disequilibrium were minimal. ATP, as the disodium salt (Pabst or Sigma), was dissolved in 3 M NaCl and neutralized to pH 7.5 in the cold. The concentration of ATP ranged from 0.01 M to 0.1 M, most experiments being at the higher concentration.

RESULTS

No significant change ($P > .15$) in the resting membrane potential was produced by the injection of 3 M KCl, immediately or up to 10 minutes (table 1). This was surprising, since an increased internal concentration of KCl was expected to lower the potential considerably (see discussion); nor did 3 M NaCl affect the membrane potential. Comparable results for squid axon have been reported recently (Grundfest, et al., '53). No

TABLE 1
Effect of injections on membrane potential

SOLUTION	MEMBRANE POTENTIAL, mV		P
	Before	After	
KCl, 3 M	84.0 ± 1.9	80.0 ± 2.1	$> .15$
NaCl, 3 M	84.6 ± 1.7	83.2 ± 1.4	.4
ATP, 0.1 M	82.1 ± 1.3	80.0 ± 1.8	.4

Mean values and standard errors are given.

TABLE 2
ATP and contraction

CONTRACTION	IMPALEMENT	
	Good	Poor
Present	2	22
Absent	13	15
% Contraction	13	60

contraction or other change of the fiber was seen after either salt.

Injection of ATP (0.01–0.1 M) also failed to alter the resting potential or, mostly, to produce a mechanical response. The fiber was observed closely during injection and, with good impalements, twitching was seen in only 2 of 15 trials (table 2). The criterion for a good impalement was the appearance of a potential of 80 mV or greater instantaneously upon penetration. Poor impalements presumably involve imperfect sealing

around the electrode and some muscle injury; and the likelihood is great that injected material would leak out at the injection site. In any event, with poor impalements response occurred in 60% of 37 trials. Moreover, wiggling the electrode in a good impalement, during injection, invariably led to prompt twitching. The muscle thus remained responsive, despite the high concentration of NaCl injected with the ATP. It appears that injected ATP does not evoke a response if it remains inside the fiber. External ATP does produce twitching, presumably by a surface action. Flowing ATP-Ringer over the muscle gives a mechanical response at the threshold

TABLE 3
External ATP

ATP CONC.	MEMBRANE POTENTIAL	CONTRACTION
<i>M</i>	<i>mV</i>	
0.000	96.7 $\pm$ 1.2	—
0.002	95.0 $\pm$ 1.0	$\pm$
0.005	93.0 $\pm$ 0.7	+
0.01	94.3 $\pm$ 1.1	++

concentration of 0.002 M ATP; but even at 0.01 M the membrane potential of actively twitching fibers was unaltered (table 3). Typical repetitive propagated action potentials (method of Nastuk and Hodgkin, '50) were recorded during the twitching; but it must be concluded that ATP does not directly evoke contraction, but simply activates the normal membrane response that leads to contraction.

DISCUSSION

Membrane potential

Hodgkin and coworkers have derived equations for the movement of passive ions through the membrane under the influence of electro-chemical gradients, while Ussing ('52) has described mathematically the transport of ions which do not

move solely as a result of the electric field and thermal agitation. An equation for the resting membrane potential which incorporates the ideas of these investigators can be formulated as follows.

$$I = \sum_n g_n (E - E_n) + \sum_p g_p (E - E_p) = 0 \quad (1)$$

where I is the ionic current density

g_n is the ionic conductance of the n th ion

E is the electromotive force in volts across the membrane

E_n is the equilibrium potential of the n th ion

g_p is the ionic conductance of an extruded ion

E_p is the electromotive force of the extruding process

Solving for E

$$E = \frac{\sum_n g_n E_n + \sum_p g_p E_p}{\sum_n g_n + \sum_p g_p} \quad (2)$$

Since

$$E_n = \frac{RT}{Z_n F} \ln c_{n1}/c_{no} \quad (3)$$

Where c_{n1} is the activity of the n th ion inside the cell

c_{no} is the activity of the n th ion outside the cell

Z_n is the valence of the n th ion

$$E = \frac{RT}{F} \left[\frac{\sum_n g_n / Z_n (\ln c_{n1}/c_{no})}{\sum_n g_n + \sum_p g_p} \right] + \frac{\sum_p g_p E_p}{\sum_p g_p + \sum_n g_n} \quad (4)$$

By definition

$$t_n = \frac{g_n}{\sum_n g_n + \sum_p g_p} \text{ and } t_p = \frac{g_p}{\sum_n g_n + \sum_p g_p} \quad (5)$$

where t_n is the transference number of the n th ion

t_p is the transference number of the extruded ion

Hence

$$E = \frac{RT}{F} \left[\sum_n t_n / Z_n (\ln c_{n1}/c_{no}) \right] + \sum_p t_p E_p \quad (6)$$

Specifying the ions involved

$$E = \frac{RT}{F} (t_k \ln \frac{K_1}{K_o} + t_{c1} \ln \frac{Cl_o}{Cl_i} + t_{Na} \ln \frac{Na_1}{Na_o}) + \sum_p t_p E_p \quad (7)$$

If the last term (involving mainly the sodium pump) is negligible, as assumed by Hodgkin, it follows that the resting membrane potential will be determined by the relative concentration of the passive ions and their respective transference numbers through the membrane. Unfortunately the latter are not known very accurately for the frog sartorius. The best fit to account for the normal resting potential would be $t_k = .6$, $t_{cl} = .35$, $t_{Na} = .05$. These values, even if not precise, imply that the bulk of the resting membrane conductance is due to the potassium and chloride ions.

Since chloride is the least concentrated of the ions in the cell (Boyle and Conway, '41), it was anticipated that the injection of NaCl or KCl would lower the potential by affecting primarily the chloride potential. The exact magnitude of the fall expected cannot be computed accurately since the distribution of the injected fluid is not known and since the transport numbers of the ions is subject to uncertainty. If the injected ions were evenly distributed in the fiber, the fall in potential, with the largest amounts of KCl or NaCl injected, should have been of the order of 20 mV; with medium amounts, 6 to 7 mV.

Some explanations of the discrepancy between theory and observation are possible. If, for example, the region of the fiber injected suddenly developed a high resistance and capacitance, so that it resembled the myelin sheath electrically, then it might be possible to record the same resting and action potential, despite a change in potential in the region of the electrode. The most likely is that the chloride conductance is considerably lower than assumed and that the outward sodium pump current is not negligible, but contributes significantly to the membrane conductance. Chloride would then add little to the resting potential, whereas the sodium pump could contribute 30-40%. There is considerable evidence suggestive of an active sodium pump in muscle.

In the isolated squid axon, where the sodium pump may be depressed, the measured membrane potential is 30% lower than the equilibrium potential for potassium (Hodgkin and Huxley, '52b); whereas the measured membrane potential of

the isolated frog sartorius is close to the K potential — as if the sodium extrusion mechanism were better maintained. The nerve also gains sodium at a faster rate than does the muscle. Moreover, the change in membrane potential for a tenfold change in external potassium should deviate from the theoretical 58 mV by an amount depending on other ion effects and on the pump term (equation 7); and the measured deviation is considerably greater for the muscle than for the nerve (Curtis and Cole, '42; Ling and Gerard, '50). Finally, in support of an active sodium pump is the finding that isolated frog skin, operating as a sodium battery, can give large and maintained ionic currents (Ussing, '52).

The sodium pump would require energy. Although Rothenberg ('50) found a Q_{10} of 1.25 for sodium exchange, chemical reactions are not excluded, for, if Na transport is by carriers, then their diffusion would be a limiting step and determine the Q_{10} (Ussing, '52). Ling ('52) has reported that sodium does not enter muscle along a concentration gradient even at 0°C. when oxygen is excluded and glycolysis blocked by iodoacetate. If, as he assumes, all significant energy pathways are inactivated under these conditions, an energy-consuming pump would be excluded. Against this assumption, however, is the report that frog muscle still produces heat and is capable of contraction upon stimulation under these very conditions (Hukuda, '31). Ordinarily, when metabolism is interfered with, the membrane potential falls part way in frog muscle (Ling and Gerard, '49b) and nerve (Shanes and Brown, '42; Lorente de Nó, '47). The metabolism-sensitive potential of muscle (A potential) constitutes the same fraction of the total potential, 30 to 40%, as here suggested due to the sodium pump.

The magnitude of the A potential was found to correlate with the concentration of high energy phosphate compounds, notably creatine phosphate and possibly ATP. The failure of injected ATP to increase potential may be due to the initial presence of a high ATP content.

Contraction

It is generally agreed that adenosine triphosphate is intimately associated with the contractile process, whether with contraction or relaxation. The failure of injected ATP to alter the mechanical state would speak against this, unless it be assumed that the added ATP produces an inconsequential change in concentration — by not reaching critical regions, by being rapidly destroyed, or by adding to a large reservoir of free ATP. This finding militates against the possibility that normal contraction depends on a sudden release of ATP from bound form. Further, externally applied ATP must be able to cause contraction (see also Buchtal et al., '44) without penetrating. A comparable situation appears valid for heart muscle (Marshall and Andrus, '53). ATP, thus, has a direct action on the cell membrane, whatever its intracellular role.

ATP elicits propagated responses of the membrane, like the tetanus from applied shocks, rather than maintained contraction and depolarization, as produced by potassium and some other agents. The only potential change with ATP is the conducted action spike, in harmony with other evidence that shortening is void of any potential sign (Taylor, '53). The localization of ATP-ase in the membrane region of the squid giant nerve fiber (Libet, '48) is suggestive in this connection, as is the ability of transmembrane currents, but not longitudinal ones in the fiber interior, to alter the CrP content of muscle fibers (Taylor and Abood, '53). Moreover, energy-rich phosphate is as concentrated in nerve (excluding myelin) as in muscle (Gerard and Tupikova, '38), despite the vast difference in energy needs for activity; CNS discharges are associated with decreased PO_4 turnover (Streicher et al., '52); and brain mitochondria, "stimulated" by electric shocks, show an inhibition of PO_4 synthesis (Abood et al., '52). Finally, an iodoacetate poisoned muscle goes into rigor as its $\sim \text{P}$ is exhausted (Lundsgaard, '30); and a glycerinized muscle remains relaxed in ATP, goes into rigor when this is washed out and relaxes

again with ATP added, although no metabolic activity is present (see Gerard and Taylor, '53, for further discussion).

Such facts suggest that ATP is related to conduction (in nerve and muscle) and to contraction as part of the machinery more than as an energy source. A fall in the ATP concentration might depend on slowed production rather than on hastened splitting and might precede, rather than follow, the functional response. Certainly, the exact role of ATP in controlling membrane activation and micelle length has not yet been clarified.

SUMMARY

1. Sodium chloride or potassium chloride, injected into single sartorius muscle fibers, does not significantly alter the resting membrane potential. From an equation relating potential to ion concentrations and transport, it appears that this result would be explained if about one-third of the membrane potential — approximately the value of the metabolism-sensitive A potential — were contributed by the activity of a sodium pump.

2. ATP, on injection, does not produce contraction nor alter membrane potential. Applied externally, it evokes typical conducted action spikes with attendant twitching. No other potential changes, nor contracture, occur. The role of ATP in membrane activation, and as part of the machinery of function, rather than as an energy source, is considered.

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LITERATURE CITED

- ABOOD, L. G., R. W. GERARD AND S. OCHS 1952 Electrical stimulation of metabolism of homogenates and particulates. *Am. J. Physiol.*, 171: 134-139.
- BERNSTEIN, J. 1902 Untersuchungen zur Thermodynamik der bioelektrischen Ströme. *Pflüg. Arch. ges. Physiol.*, 92: 521-562.
- BOYLE, P. J., AND E. J. CONWAY 1941 Potassium accumulation in muscle and associated changes. *J. Physiol.*, 100: 1-63.

- BUCHTAL, F., A. DEUTCH AND G. G. KNAPPEIS 1944 Release of contraction and changes in birefringence caused by adenosine triphosphate in isolated cross striated muscle fibers. *Acta Physiol. Scand.*, *8*: 271-287.
- GERARD, R. W., AND R. E. TAYLOR 1953 Muscle and nerve-physiologic orientation. *Am. J. Med.*, *15*: 83-98.
- GERARD, R. W., AND N. TUPIKOVA 1938 Creatine in nerve, muscle and brain. *J. Cell. and Comp. Physiol.*, *12*: 325-360.
- GRUNDFEST, H., M. ALTAMIRANO-ORREGO, C-Y. KAO AND D. NACHMANSOHN 1953 Resting and action potentials of squid axons with internal environment altered by microinjection. *Fed. Proc.*, *12*: 58.
- HODGKIN, A. L. 1951 The ionic basis of electrical activity in nerve and muscle. *Biol. Rev.*, *26*: 339-409.
- HODGKIN, A. L., AND A. F. HUXLEY 1952a Currents carried by sodium and potassium ions through the membrane of the giant axon of *Loligo*. *J. Physiol.*, *116*: 449-472.
- 1952b A quantitative description of membrane current and its application to conduction and excitation in nerve. *J. Physiol.*, *117*: 500-544.
- HUKUDA, K. 1931 The energy liberated in total exhaustion of frog's muscle. *J. Physiol.*, *72*: 438-442.
- LEVI, H., AND H. H. USSING 1948 The exchange of sodium and chloride ions across the fibre membrane of the isolated frog sartorius. *Acta. Physiol. Scand.*, *16*: 232-249.
- LIBET, B. 1948 Adenosinetriphosphatase (ATP-ase) in nerve. *Fed. Proc.*, *7*: 72.
- LING, G. N. 1952 The role of phosphate in the maintenance of the resting potential and selective ionic accumulation in frog muscle cells. *Phosphorus Metabolism*, *2*: 748-795, The Johns Hopkins Press, Baltimore.
- LING, G., AND R. W. GERARD 1949a The normal membrane potential of frog sartorius fibers. *J. Cell. and Comp. Physiol.*, *34*: 383-396.
- 1949b The membrane potential and metabolism of muscle fibers. *J. Cell. and Comp. Physiol.*, *34*: 413-438.
- 1950 External potassium and the membrane potential of single muscle fibers. *Nature*, *165*: 113-114.
- LORENTE DE NÓ, R. 1947 A study of nerve physiology. Studies from the Rockefeller Institute for Medical Research, vol. 131, New York.
- LUNDSGAARD, E. 1930 Weitere Untersuchungen über Muskelkontraktion ohne Milchsäurebildung. *Biochem. Z.*, *227*: 51-83.
- MARSHALL, J. M., AND E. C. ANDRUS 1953 Comparison of effects of various phosphate compounds and aluminum silicate on isolated frog heart. *Proc. Soc. Exp. Biol. and Med.*, *82*: 228-231.
- NASTUK, W. L., AND A. L. HODGKIN 1950 The electrical activity of single muscle fibers. *J. Cell. and Comp. Physiol.*, *35*: 39-74.
- ROTHENBERG, M. A. 1950 Studies on permeability in relation to nerve function. II. Ionic movements across axonal membranes. *Bioch. et Biophys. Acta*, *4*: 96-114.
- SHANES, A. M., AND D. E. S. BROWN 1942 The effect of metabolic inhibitors on the resting potential of frog nerve. *J. Cell. and Comp. Physiol.*, *19*: 1-13.

- STREICHER, E., L. G. ABOOD AND R. W. GERARD 1952 Phosphorus turnover in stimulated and depressed brain. *Am. J. Physiol.*, 171: 771.
- TAYLOR, R. E. 1953 The contractile process is not associated with potential changes. *J. Cell. and Comp. Physiol.*, 42: 103-124.
- TAYLOR, R. E., AND L. G. ABOOD 1953 Unpublished.
- USSING, H. H. 1949 Transport of ions across cellular membranes. *Physiol. Rev.*, 29: 127-155.
- 1952 Some aspects of the application of tracers in permeability studies. *Adv. Enz.*, 13: 21-65.

SUMMATION, INHIBITION, AND PROPRIOCEPTIVE
REINFORCEMENT UNDER CONDITIONS OF
STIMULATION OF THE MOTOR CORTEX
AND THEIR INFLUENCE ON THE
ACTIVITY OF SINGLE
MOTOR UNITS¹

E. GELLHORN, C. M. RIGGLE, AND H. M. BALLIN
Laboratory of Neurophysiology Department of Physiology
University of Minnesota

Following a study of the relation between various parameters of stimulation of the motor cortex and the discharge of single motor units (Hyde, Riggle and Gellhorn, '54), it was thought to be of interest to study motor units under the simultaneous influence of stimulation of the motor area and proprioceptive reflexes. The far-reaching influence of this interaction on the intensity and the pattern of movement has been reported previously (Gellhorn, '49; Gellhorn and Johnson, '50). Additional experiments concerned the summation induced by stimulation of two cortical foci from which the same muscle could be activated. Finally the antagonistic relation of biceps and triceps complexes (Bosma and Gellhorn, '47), demonstrated on stimulation of the biceps and triceps area of the motor cortex, served as a basis for the study of cortical inhibition and its effect on single unit discharges.

The experiments were performed on 5 macaques in Dial-urethane anesthesia. Concerning the methods see the preceding paper (Hyde, Riggle and Gellhorn, '54).

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Proprioceptive reinforcement and unit discharges

It has been found previously that proprioceptive reinforcement is maximal under conditions of fixation of a joint when the muscles which give rise to proprioceptive impulses are stretched and contract isometrically on cortical stimulation. On the other hand, the proprioceptive effect is minimal when the joint is fixated at an angle at which the muscles are slack before cortical stimulation. Experiments on the triceps were therefore performed with the elbow fixed at acute and obtuse angles to provide conditions of maximal and minimal proprioceptive reinforcement respectively. The position of shoulder and wrist joints remained unchanged.

Experiments 1-7 of table 1 show the effect of proprioceptive reinforcement when a site in the cortical triceps area is stimulated. The gross response of the muscle was recorded as the electromyogram (EMG), the amplitude of which has been shown to be quantitatively related to muscle tension (Loofbourrow, '48). The total number of activated individual units recorded and the maximal rate of any of these units discharging during the period of optimal response of the muscle are also listed. The results are uniform, showing that under the influence of proprioceptive reinforcement the number of discharging units increases. This change is paralleled by an increase in the amplitude of the EMG. In most of the experiments of table 1 the intensity of stimulation was so chosen that under conditions of minimal proprioceptive reinforcement no single unit activity was recorded with the microelectrodes, and even the gross response (EMG) was either very small or absent. This condition made the action of proprioceptive facilitation very conspicuous. However, even in cases in which several units discharged on cortical stimulation in the "minimal" position (illustrated in experiment 5 of table 1), the action of proprioceptive facilitation appeared as an increase in the number of activated units and in the maximal rate of firing. Table 1 also illustrates the validity of these rules for the tibialis anterior muscle in

TABLE 1

Effect of proprioceptive impulses (fixation) on unit discharges resulting from the stimulation of the motor cortex

NO.	INTEN- SITY	FRE- QUENCY	POSI- TION	GROSS AMPLIT.	TOTAL NO. OF UNITS	MAX. UNIT RATE	REMARKS
	<i>volts</i>	<i>c/s</i>		<i>mm</i>		<i>c/s</i>	
I. Experiments on triceps brachii							
1	3.0	83	A	0	0	0	In all positions shoulder abducted and retracted (angle 60° with length axis of the body).
	3.0	83	B	22	3	30	
	3.0	83	A	3	0	0	
2	3.0	53	A	0	0	0	A, elbow 150°; wrist 20° dorsi- flexion.
	3.0	53	B	15	3	25	
	3.0	53	A	3	0	0	
3	3.5	83	A	5	0	0	B, same as A, but elbow 75°.
	3.5	83	B	17	3	27	
	3.5	83	A	0	0	0	
4	3.5	53	A	1	0	0	C, same as A, but wrist 100° dorsi- flexion.
	3.5	53	B	15	3	25	
	3.5	53	A	1	0	0	
5	4.2	83	A	7	3	27	
	4.2	83	B	20	5	39	
	4.2	83	A	6	2	23	
6	4.2	53	A	4	0	0	
	4.2	53	B	15	3	28	
	4.2	53	A	3	0	0	
7	4.2	33	A	7	0	0	
	4.2	33	B	14	4	30	
	4.2	33	A	3	0	0	
8	4.2	33	A	3	0	0	
	4.2	33	C	5	3	35	
	4.2	33	A	2	0	0	
9	4.2	53	B		3	28	
	4.2	53	A		0	0	
	4.2	53	C		4	45	
II. Experiments on the tibialis anticus							
10	3.5	53	A	1	0	0	A, hip 80°, knee 90°, ankle 60°.
	3.5	53	B	14	1	22	
	3.5	53	A	1	0	0	
11	4.2	53	A	1	0	0	B, hip 80°, knee 90°, ankle 150°.
	4.2	53	B	12	5	35	
	4.2	53	A	0	..	..	
12	4.2	33	A	3	0	0	
	4.2	33	B	4	2	35	
	4.2	33	A	3	..	..	

which the degree of proprioceptive reinforcement is augmented by fixation of the foot in plantar flexion.

Finally a special case of proprioceptively induced summation is illustrated in experiments 8 and 9 of table 1. It was shown previously that stimulation of the biceps and triceps foci of the motor area elicits complex patterns of movement. Under conditions of fixation this pattern appears in the form of selectively activated groups of muscles. The intensity and distribution of this pattern are indicated by the appearance and particularly the amplitude of the EMG's in various muscles. Thus it was found that activation of a triceps focus was associated with the development of tension and the appearance of action potentials in the flexor carpi muscles. Triceps and flexor carpi muscles, and a great many additional muscles (see Gellhorn and Johnson, '50), form a functional unit, the triceps complex, within which proprioceptive reflexes play an important role. Thus fixation of the triceps at an acute angle increases the response of this muscle as well as that of the flexor carpi to a standard stimulus applied to the motor cortex, although the fixation of the wrist remains unchanged in this experiment. Similarly it was noted that fixation of the wrist in marked dorsiflexion increases the response of the flexor carpi *and* triceps to cortical stimulation, although the position of the elbow joint remains the same. The influence on unit potentials of these factors is illustrated in nos. 8 and 9 of table 1. Fixation in dorsiflexion of the wrist is shown to increase the amplitude of the EMG of the triceps and the number and maximal rate of the discharging units in the triceps muscle.

Table 2 presents additional data on the influence of proprioceptive reinforcement on unit discharges in a series of experiments in which the motor cortex was stimulated at three different frequencies. It is evident from the results that with fixation of the ankle at a greater degree of plantar flexion the number of discharging units increases. The number of spikes recorded during one second of the optimal period of response is likewise augmented. This number de-

depends on the number of discharging units and the number of discharges of each unit during this period. Here again these numbers are more significantly related to the central excitation than is the maximal rate of any discharging unit (cf. Hyde, Riggle and Gellhorn, '53). This rate is practically the same as the stimulus frequency in the first two experiments and remains unchanged under conditions of increased proprioceptive excitation. In the third experiment, however, the maximal rate of discharge as well as the total number of spikes and the number of discharging units increases with proprioceptive facilitation.

TABLE 2

Influence of proprioception on single unit response to cortical stimulation of varying frequencies

INTENSITY	FREQUENCY	ANKLE	TOTAL NO. OF UNITS RESPONDING	MAX. UNIT RATE	TOTAL NO. OF SPIKES IN 1" ¹
<i>volts</i>	<i>c/s</i>	<i>°</i>		<i>c/s</i>	
3.0	27	70	2	28	17
3.0	27	140	4	28	42
3.0	53	70	2	56	27
3.0	53	140	4	58	57
3.0	102	70	4	51	63
3.0	102	140	6	72	83

¹ Selected from the period of optimal response. The records were obtained from the tibialis anticus; the degrees of plantar flexion are indicated in the table in the column "Ankle."

*Spatial summation under conditions of stimulation
of the motor area and unit discharges*

Since proprioceptive facilitation is only a special case of summation, it was deemed advisable to perform additional experiments under somewhat different conditions of summation. For this purpose two foci in the motor cortex, which when stimulated singly induced a contraction of a certain muscle, were stimulated singly and in combination in order to determine the gross response (EMG), the total number of discharging units, and the maximal rate of the fastest discharg-

ing unit under these conditions. The results are illustrated for the biceps, triceps, and extensor carpi radialis in table 3. The table shows unit and gross responses 3.5 and 6.5 seconds after the stimulus A. When two stimuli, A and B, were used, B was applied 4.4 seconds after the onset of A. The response recorded in the right part of the table (6.5 seconds after the beginning of stimulation) was obtained during the simultaneous excitation of both cortical foci.²

The results were uniform. In all but one experiment the gross response (amplitude of the EMG) was greater when the two stimuli were applied than when the A or the B stimulus was used. It was also found that in all experiments summation resulted in recruitment, since the number of discharging units was increased. Consequently the number of spikes recorded during one second of the period of optimal contraction was augmented.³ However, it was somewhat unexpected that in these experiments the maximal rate of the fastest unit was regularly increased. This is seen especially in the experiments on the extensor carpi and triceps muscles. Nevertheless, it should be pointed out that the maximal rate of firing was only a fraction of the stimulus frequency even under conditions of cortically induced summation.

*Cortically induced inhibition and its influence
on unit discharges*

The last series of experiments concerns the action of cortically induced inhibition on the unit discharges of biceps or triceps muscles. In this work biceps and triceps foci were stimulated singly or in combination. When combined, the second stimulus followed the onset of the first by several seconds, the stimulation of the first focus being continued. Gross responses were recorded from both muscles, but the unit discharges were recorded only from the muscle activated

² There are some slight temporal deviations in the application of the two stimuli in the experiments on the triceps muscle (see table 3).

³ These numbers were omitted from the table.

TABLE 3

Effect of spatial cortical summation on gross and unit responses of skeletal muscles

A ¹ INT.	B INT.	B FREQ.	RESPONSE 3.5 SEC. AFTER ONSET OF A			RESPONSE 6.5 SEC. AFTER ONSET OF A AND/OR 2.1 SEC. AFTER B		
			Total no. of units	Max. unit rate	Gross amplit.	Total no. of units	Max. unit rate	Gross amplit.
volts	volts	c/s	per sec.		mm	per sec.		mm
1. Biceps brachii								
3.2	..	..	0	0	12	1	1	11
3.2	2.3	58	0	0	10	5	25	15
..	2.3	58	..	..	..	0	0	2
3.1	..	..	0	0	13	0	0	11
3.1	2.3	58	0	0	12	5	31	22
..	2.3	58	..	..	..	0	0	0
3.1	..	..	0	0	10	0	0	10
3.1	2.3	71	0	0	13	4	16	23
..	2.3	71	..	..	..	0	0	0
2. Extensor carpi radialis								
3.0	..	..	7	29	18	5	21	15
3.0	3.2	82	6	25	18	7	34	15
..	3.2	82	..	..	..	2	20	2
1.2	..	..	0	0	0	4	25	1
1.2	2.6	82	0	0	0	6	40	5
..	2.6	82	..	..	..	1	5	0
1.2	..	..	0	0	0	4	15	0
1.2	3.0	82	3	15	0	6	40	10
..	3.0	82	..	..	..	3	20	1
..	1.9	82	..	..	..	0	0	0
1.2	1.9	82	0	0	0	6	30	2
1.2	..	..	0	0	0	0	0	0
1.2	..	..	0	0	0	0	0	0
1.2	1.9	82	0	0	0	8	52	7
..	1.9	82	..	..	..	5	40	5
3. Triceps brachii								
3.5	..	..	0	0	0	1	22	7
3.5	3.8	82	0	0	0	4	50	10
..	3.8	82	..	..	..	3	30	3
			RESPONSE 4 SEC. AFTER START OF A STIMULUS			RESPONSE 6.5 SEC. AFTER START OF A STIMULUS AND/ OR 2.2 SEC. AFTER START OF B STIMULUS		
3.0	..	..	0	0	0	4	38	13
3.0	3.8	82	0	0	0	6	56	20
..	3.8	82	..	..	..	4	35	1

¹ Placement of cortical electrodes: Biceps expts.: A, 24 mm lat., 1 mm anterior to central fissure; B, 20 mm lat., 1 mm ant. Extensor carpi expts.: A, 20 mm lat., 1 mm ant.; the B electrode was 15 mm lat., 1 mm ant. in the first group, 24 mm lat., 3 mm ant. in the second and third groups, and 24 mm lat., 1 mm ant. in the last group.

The frequency of stimulation for the A stimulus was 83/sec. in the biceps and extensor carpi expts. and 53/sec. in the triceps expts.

by the first stimulus. The experiments show uniformly that cortically induced inhibition of the biceps by stimulation of a triceps focus in the motor cortex leads to a reduction in the gross amplitude of the EMG of the biceps, a reduction in the number of discharging units and therefore of the total spikes recorded from all discharging units during the period of optimal inhibition,⁴ and also to a diminution in the maximal rate of the fastest discharging unit (table 4). Similar results were obtained in inhibiting the contraction of the triceps muscle through stimulation of a cortical biceps focus. It may be inferred that spatial summation and inhibition induced by the interaction of two similar and two antagonistic cortical foci act on the single motor units of the final common path in an opposite manner.

COMMENT

The experiments show that proprioceptive facilitation and spatial cortical summation increase discharges from the motor cortex primarily by increasing the number of discharging units and thereby by augmenting the total number of unit discharges per unit of time. Individual units gradually increase their rate of discharge during the period of stimulation until the spikes appear at a rather constant rate, which is ordinarily below that of the stimulus frequency. Under conditions of proprioceptive facilitation and particularly under the combined influence of stimulation of two cortical foci this maximal rate of discharge is also increased.

The cortically induced inhibition results in a decruitment and in a diminished rate of the fastest discharging unit.

Summation and inhibition induced by stimulation of appropriate foci in the motor cortex may influence the same single motor unit in the biceps or triceps muscles, although their cortical foci are widely separated. To what extent this interaction takes place at a spinal as well as at a supraspinal level has not been determined.

⁴ These numbers are omitted from table 4.

The experimental results presented in this paper are in general agreement with the Adrian-Bronk law (see Gellhorn, '53).

TABLE 4

The effect of cortical inhibition on unit responses

STIMULATION				RESPONSE 2.6 SEC. AFTER ONSET OF BICEPS STIM.			RESPONSE 5.8 SEC. AFTER ONSET OF BICEPS STIM. AND/OR 3.0 SEC. AFTER TRICEPS STIM.			
Biceps		Triceps		Total no. of units	Max. unit rate	Gross amplit.	Total no. of units	Max. unit rate	Biceps gross amplit.	Triceps gross amplit.
Foci	Foci									
V	c/s	V	c/s							
I. Biceps recorded ¹										
				per sec.	mm		per sec.	mm	mm	
2.4	66	..		4	27	10	7	45	12	1
2.4	66	3.5	82	4	20	10	1	8	5	6
2.4	66	..		4	20	8	5	34	11	0
2.1	66	..		1	14	5	6	35	9	1
2.1	66	3.5	82	4	38	7	2	9	4	6
2.1	66	..		2	10	6	6	43	9	1
1.8	66	..		0	0	1	4	13	9	1
1.8	66	3.5	82	0	0	4	3	9	3	4
1.8	66	..		0	0	3	4	17	9	1
2.1	66	..		4	21	7	5	46	11	2
2.1	66	4.0	82	6	27	7	3	8	4	3
2.1	66	..		0	0	2	3	14	9	1
II. Triceps recorded										
STIMULATION				RESPONSE 3.5 SEC. AFTER ONSET OF TRICEPS STIM.			RESPONSE 6.5 SEC. AFTER ONSET OF TRICEPS STIM. AND/OR 2.7 SEC. AFTER BICEPS STIM.			
Triceps		Biceps								
Foci	Foci									
V	c/s	V	c/s							
3.8	53	2.4	82	4	25	14	2	9	8	7
..	..	2.4	82	..	..	..	0	0	5	0
3.8	53	..	..	1	2	1	5	34	6	9
3.8	53	2.4	82	0	0	1	0	0	7	0
4.2	83	..	..	3	27	1	3	29	2	2
4.2	83	3.0	82	4	35	3	0	0	5	0
		3.0	82	0	..	..	0	0	4	0

¹ Biceps and triceps foci 15 and 11 mm (expt. I) and 19 and 11 mm (expt. II) lateral to midline and 1 mm anterior to central fissure.

SUMMARY

Experiments are reported on the macaque concerning the interaction between proprioceptively induced reflexes and stimulation of the motor cortex as these affect single unit discharges recorded from a muscle. In addition, summation and inhibition induced by stimulation of appropriate foci of the motor area have been studied in their effect on unit discharges. The results were as follows:

1. Proprioceptive reinforcement of cortically induced movements is accompanied by a recruitment of more units, so that the total number of spikes (unit discharges) which appear during a given period of the optimal response (indicated by the greatest amplitude of the electromyogram) is augmented. However, the maximal rate of the fastest recorded unit is often unchanged and in most instances far below the stimulation frequency, particularly if the latter is 80 to 100/sec.

2. If summation results from stimulation of two cortical foci affecting the same muscle, there is not only recruitment but also an increase in the maximal rate of unit discharges. Conversely, it is found that during cortically induced inhibition (stimulation of the biceps focus during a contraction initiated by stimulation of a triceps focus and vice versa) the number of discharging units decreases, and the maximal rate of any discharging unit is diminished.

LITERATURE CITED

- BOSMA, J. F., AND E. GELLHORN 1947 The organization of the motor cortex of the monkey based on electromyographic studies. *Brain*, 70: 127-244.
- GELLHORN, E. 1949 Proprioception and the motor cortex. *Brain*, 72: 35-62.
- 1953 *Physiological Foundations of Neurology and Psychiatry*. University of Minnesota Press.
- GELLHORN, E., AND D. A. JOHNSON 1950 Further studies on the role of proprioception in cortically induced movements of the foreleg in the monkey. *Brain*, 73: 513-531.
- HYDE, J., C. M. RIGGLE AND E. GELLHORN 1954 The dependence of unit discharges on frequency and intensity of stimulation of the motor cortex in the macaque. *J. Cell. and Comp. Physiol.*, 43: 293-310.
- LOOFBOURROW, G. N. 1948 Electrographic evaluation of mechanical response in mammalian skeletal muscle in different condition. *J. Neurophysiol.*, 11: 153-168.

LUMINOUS BACTERIA AS A RADIOBIOLOGICAL TEST ORGANISM^{1,2}

G. HOYT WHIPPLE

*Department of Radiation Biology
University of Rochester School of Medicine and Dentistry
Rochester, New York*

TWO FIGURES

INTRODUCTION

The peculiar advantage offered by luminous bacteria for biological investigations has been exploited for a number of purposes (Johnson, '51) and it has been pointed out that these bacteria are highly suitable for radiobiological investigations (Harvey, '52). There has been work on the effects of ultra-violet light on luminous bacteria (Giese, '41), but with the exception of a qualitative study of the effect of various amounts of radon dissolved in culture media on the luminescence of bacteria grown on these media (Rerabek and Hykesova, '37), and an investigation of the effect of various x-ray doses on the colony-forming ability of *Achromobacter fischeri* (Lorenz and Henshaw, '41), there is almost nothing to be found. The purpose of this investigation was to explore the suitability of one species of luminous bacteria for radiobiological studies.

EXPERIMENTAL

The bacteria used in this work were grown from a culture of *Achromobacter fischeri*, strain of F. H. Johnson, obtained from

¹ This paper is based on work performed under contract with the United States Atomic Energy Commission at The University of Rochester, Atomic Energy Project, Rochester, New York.

² This paper is based on a thesis submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

the American Type Culture Collection. The bacteria were grown on the following medium: distilled water, 1,000 cm³; NaCl, 30 gm; Difco Bacto-agar, 20 gm; Parke, Davis bacteriologic peptone, 10 gm; glycerine, 10 cm³; CaCO₃, 10 gm; and frozen ocean perch comminuted to a thin paste, 10 gm. This medium differs from those commonly used for marine luminous bacteria principally in the addition of fish, which appeared to reduce the incidence of luminous decline in the colony. The medium was poured into petri dishes, allowed to cool, and stored at 4°C. until used.

At least one fresh plate was inoculated lightly every day to maintain the colony. The plates were incubated at 20° ± 1°C.

The bacterial suspensions were prepared from plates which had been heavily inoculated expressly for this purpose 24 hours previously. Suspensions were prepared and maintained in a non-nutrient, buffered sea water solution, "BS-2," which has the following composition: NaCl, 15 gm; Na₂HPO₄, 14.2 gm; NaH₂PO₄·H₂O, 3.45 gm; CaCl₂·2H₂O, 0.40 gm; and distilled water to make one liter. If necessary, the pH was adjusted to 7.2, and the precipitate which formed after an hour or two was filtered off.

The bacteria were brushed up from the culture plate in a few cubic centimeters of chilled BS-2 and were centrifuged and resuspended in fresh BS-2 three times at 9°C. The third packed volume was suspended and diluted to a concentration of 1.5×10^8 bacteria per cubic centimeter in chilled BS-2. The bacterial concentration was determined by direct count in a Petroff-Hausser chamber.

The final suspension was poured into a separatory funnel where it was stirred continuously with a motor driven glass stirring rod. Five cubic centimeters of this suspension were then run into each of ten 10 cm³ lusteroid centrifuge tubes. These tubes were stoppered and stored at 9°C. until they were used.

The lusteroid tubes containing the suspensions to be irradiated were placed in a phantom consisting of a lucite box 20 cm × 16 cm × 20 cm filled with water and ice. Each suspension

was continuously aerated with a stream of bubbles from a fine capillary inserted in the tube.

Control suspensions were kept in a beaker of ice and water for the same time the irradiated suspensions were in the phantom, and were aerated in the same manner.

Irradiations were made with a General Electric million volt industrial x-ray unit, Model RES-1000. The machine was run at 1,000 Kvp and 3.0 milliamperes. No filtration was used beyond that inherent in the machine. The radiation so obtained had a half value layer of 3.3 mm of lead. The front surface of the water phantom was placed 80 cm from the target and the dose rate was about 100 r per minute at a depth of 1.6 cm, where the lusteroid tubes were placed.

The dose measurements were made by placing a Victoreen 250 r thimble chamber in the water phantom in the same position in which the suspension tubes were to be placed. An ionization chamber was placed directly in front of the phantom. The x-ray generator was turned on for about two minutes and a recording was made of the ionization chamber current. After this exposure, the Victoreen chamber was read. This procedure was carried out several times before each irradiation in order to obtain a calibration constant for the ionization chamber recording. When this preliminary had been completed, the lusteroid tubes containing the suspensions were placed in the water phantom and a recording of the ionization chamber current was made during the irradiation. The total dose delivered to the suspensions was then obtained by measuring the area under the recording and converting it into roentgens with the calibration constant.

After an irradiation was completed, the aeration capillaries were removed from all tubes and the tubes were stoppered and stored overnight at 9°C. The capillaries which had been in irradiated suspensions were destroyed so that they could not be used again.

Twenty-four hours after the bacteria had been taken from the culture plate, all suspensions were transferred individually to 13 × 100 mm glass tubes, carefully selected for uniformity

of diameter and wall thickness. All the lusteroid tubes were discarded and were not used again. Aeration capillaries were inserted into each photometer tube and all the tubes were placed in a 20°C. water bath and connected to an air manifold. The time at which aeration in the 20°C. water bath was started was taken as $t = 0$ in plotting the light intensities.

One hundred minutes after aeration was started, 0.1 cm³ of 0.4 M glucose solution was introduced into each tube. It had been determined that this amount of glucose gave the maximum total light output under the experimental conditions.

The luminous intensity of each suspension was measured according to a standard time schedule by removing the tubes one at a time from the water bath and inserting them into the photometer. The laboratory temperature was maintained at $20^\circ \pm 1^\circ\text{C}$. and the brief interruption of the air supply to the suspension had no appreciable influence on the light output.

An RCA type 5819 photomultiplier tube was used in the photometer. The overall sensitivity of the photometer was checked before and after each set of readings with a luminous standard prepared with strontium-90 by the United States Radium Corporation expressly for this purpose. The particular standard used, S₃, had a nominal luminous intensity of about one microlambert.

The luminous intensity of each suspension was plotted as a function of time, as shown for a typical experiment in figure 1. The area under each of these curves, from the time glucose was added at $t = 100$ minutes until the arbitrary termination of the luminescence measurements at $t = 600$ minutes was measured with a planimeter. The light emitted during this 500-minute interval, which is referred to as the "500-minute light emission," was expressed in S₃-minutes, the amount of light emitted by the luminous standard in one minute.

RESULTS

The 500-minute light emissions of the control suspensions are given in table 1. It is evident that the light output varied considerably among the various runs. The light emission fell

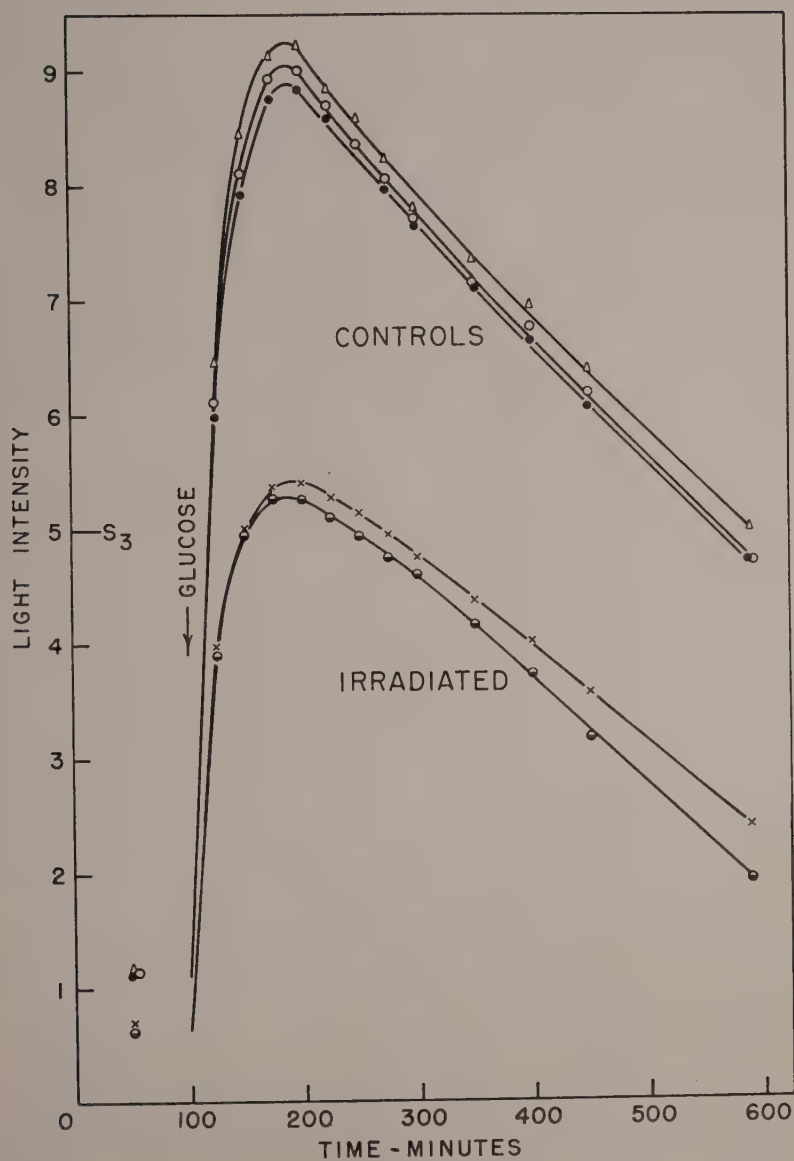


Fig. 1 Light intensity vs. time for a typical x-ray exposure.

each time a new batch of medium was introduced and gradually returned to a high level.

The data for the irradiated suspensions are given in table 2. The first column gives the number of the run, which corresponds to that given in table 1. The runs omitted from table 2 did not receive x-irradiation. The second column is the dose in

TABLE 1
Total light emission of the control suspensions

RUN	500-MINUTE LIGHT EMISSION (I_0)		AGE OF CULTURE MEDIUM
	mg-min.	%	days
1	1145	± 4.4	24
2	1324	± 5.4	28
3	507	± 8.4	8
4	576	± 5.0	11
5	677	± 4.4	14
6	1015	± 5.4	30
7	1322	± 1.6	33
8	375	± 9.2	9
9	399	± 8.1	14

TABLE 2
Data for the bacterial suspensions receiving x rays

RUN	DOSE r	I/I_0	STANDARD DEVIATION
			%
2	1,570	0.529	5.4
	3,590	0.219	
3	2,470	0.400	8.4
	4,950	0.144	
5	1,670	0.580	4.4
	3,660	0.258	
8	645	0.710	9.2
	6,610	0.095	
9	484	0.801	8.1
	1,080	0.590	

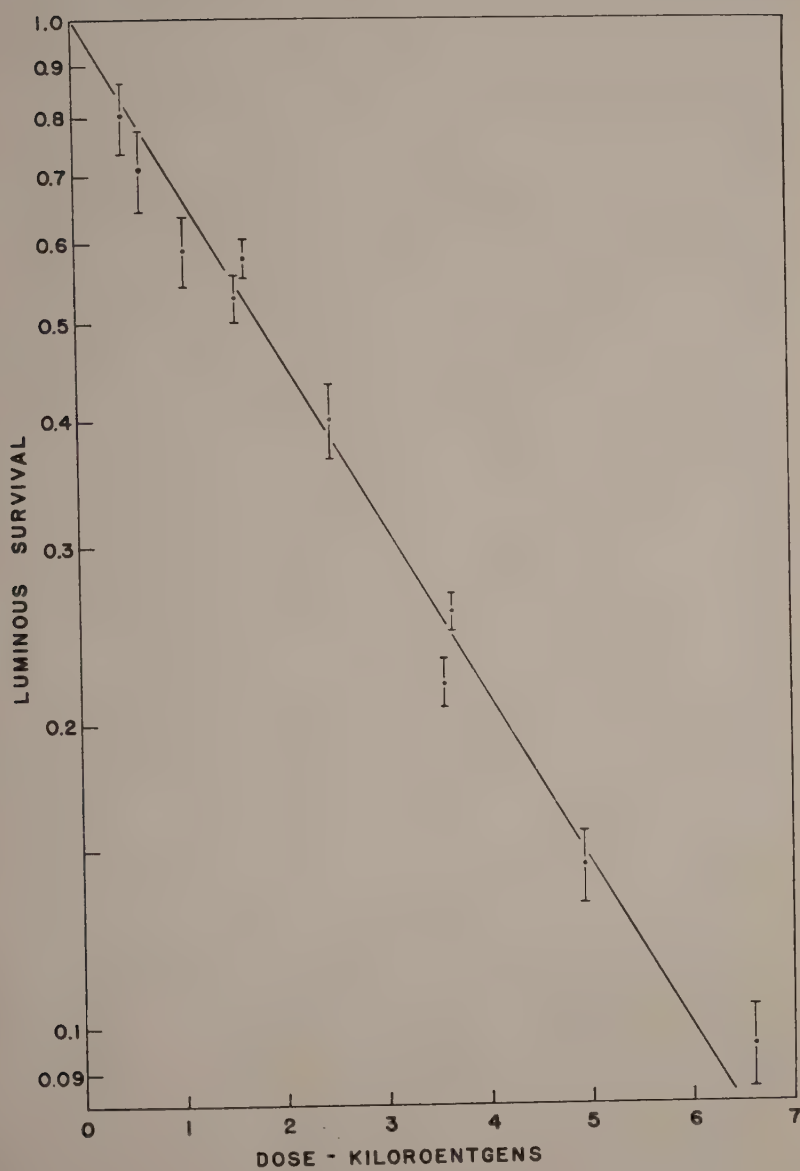


Fig. 2 Luminous survival of *A. fischeri* vs. x-ray dose.

roentgens received by the bacterial suspension. The third column gives the ratio of the 500-minute light emission from the irradiated suspensions to that from the control suspensions. This ratio is referred to as the "luminous survival." Duplicate suspension tubes were irradiated at the same time and the figures in the third column are the averages of these two values. The last column gives the standard deviation of the 6 control suspensions. In every case the individual luminous survival figures of the duplicate suspensions fell comfortably within these confidence limits.

The luminous survival data in table 2 are plotted as a function of radiation dose in figure 2. The relation is exponential within the limits of experimental error.

DISCUSSION

In spite of the considerable variation of the light output of the control suspensions from one run to the next, as shown in table 1, the radiosensitivity, as indicated by the luminous survival, appeared to remain constant within the experimental error.

The simple exponential relation which has been found in this work to exist between luminous survival and x-ray dose resembles that which has been found to exist between survival and dose for *A. fischeri* (Lorenz and Henshaw, '41) and for a number of other microorganisms (Fram, Proctor and Dunn, '50).

The x-ray dose which reduced the light output of the irradiated suspensions to 50% of the control suspensions is shown by figure 2 to be about 1800 r, while the dose which reduced the colony-forming ability, or survival, of the same organism as determined by Lorenz and Henshaw is about 3700 r. The many differences between the methods by which these two figures were obtained make any comparison between them uncertain.

A. fischeri appear to be quite satisfactory for radiobiological investigations because they can be maintained and prepared for use with relative ease and because their luminescence offers

a convenient index of biological function. This index is continuously available and, under proper experimental conditions could be observed during, as well as after irradiation.

SUMMARY

1. Experimental procedures are described for preparing suspensions of luminous bacteria (*A. fischeri*) and observing the light emission of irradiated and control suspensions. The method gives results which are reproducible to within about 10%.

2. The relation between "luminous survival" and x-ray dose, by the method described, is exponential, with a half-survival dose of about 1800 r.

3. The method appears to be a useful and convenient one for radiobiological investigations.

ACKNOWLEDGMENT

This work was undertaken at the suggestion of Dr. J. W. Howland, and would have been impossible without his sympathetic support. The choice of *A. fischeri* as the test organism was made at the suggestion of Dr. W. B. Mason, who with Dr. M. L. Ingram, gave invaluable assistance and encouragement during the development of the techniques used with these bacteria.

LITERATURE CITED

- FRAM, H., B. E. PROCTOR AND C. G. DUNN 1950 Effects of x-rays produced at 50 kilovolts on different species of bacteria. *J. Bact.*, 60: 263.
- GIESE, A. C. 1941 Effects of ultraviolet radiations on luminescence and respiration of *Achromobacter fischeri*. *J. Cell. and Comp. Physiol.*, 17: 203.
- HARVEY, E. N. 1952 Bioluminescence. Chapter I. New York Academic Press.
- JOHNSON, F. H. 1951 Luminous Bacteria. In C. H. Werkman and P. W. Wilson, Bacterial Physiology. Chapter XX. New York Academic Press.
- LORENZ, K. P., AND P. S. HENSHAW 1941 Radiobiologic action and the killing effects of x-rays on *Achromobacter fischeri*. *Radiology*, 36: 471.
- RERABEK, J., AND D. E. HYKESOVA 1937 Wirkung der Radiumemanation auf das Leuchten des Protoplasmas. (Versuche mit dem Leuchtbakterium *Vibrio phosphorescens*.) *Protoplasma*, 28: 360.

THE EFFECT OF CHANGING TEMPERATURE UPON THE TIME COURSE OF ACTIVE AND OF PASSIVE LENGTHENING OF RABBIT SMOOTH MUSCLE

TRAKUL KITISIN AND A. S. GILSON

*Departments of Physiology, Faculty of Medicine and Siriraj Hospital, Bangkok,
Thailand, and Washington University, School of Medicine,
St. Louis, Missouri*

THREE FIGURES

Recent theories of muscular contraction have placed considerable importance on the part played by passive visco-elastic factors in the determination of the time course of the response to stimulation. Bozler ('41), for example, has reported that the time-course of isometric relaxation agrees exactly with that of the decay of tension after stretch. He concludes that relaxation is controlled by the viscous properties of the contractile elements. Schoepfle (personal communication) has found that when the neck retractor (a skeletal muscle) of the turtle is cooled it shows a slowing of the isotonic twitch relaxation ("active" lengthening) but fails to show any significant change in the time-course of lengthening when a load is added to the resting, unstimulated muscle ("passive" lengthening). The present paper reports experiments undertaken to investigate the effects of temperature upon active and passive isotonic lengthening of a smooth muscle preparation.

Smooth muscle strips were obtained by isolating longitudinal muscle from the stomach of the rabbit. The strips were about 20 mm in length and about 5 mm in width. They were suspended in a bath of oxygenated Kreb's solution ('32) and allowed to remain at room temperature and supporting a load of 1 or 2 gm for about two hours. A fine tinsel wire

was attached to each end of the muscle and led to the stimulator. The latter delivered condenser discharges having a time constant of 10 msec. A hand key was used for stimulation and permitted repetitive stimulation at a fairly regular frequency of about 5 per second. For the brief periods of stimulation only, the fluid was quickly drained from around the muscle and at the completion of the stimulation the fluid was quickly restored. The fluid was often nearly or completely restored to the muscle by the end of the period of mechanical latency. The isotonic responses were recorded on smoked paper by a light lever. For loading the muscle to produce the passive stretch, 1 or 2 gm were added to the muscle load in addition to the steady load which the muscle lifted during its contraction.

Contraction, in response to several rapidly repeated shocks, yielded curves with the general configuration of those from skeletal muscle resembling either the twitch or brief tetanus response. As is true for skeletal muscle much of the relaxation curve follows the time-course of a simple exponential function. The exponential part of the curve may begin near the inflection on the falling limb of the curve and so describes roughly two-thirds of the lengthening process. When the muscle strip was stretched by the added load the resulting lengthening followed a time-course which is conveniently regarded as being made up of three parts. The first of these can be considered as instantaneous insofar as it can be measured from the records made at the drum speeds employed. Any importance of passive changes of this order of quickness was therefore neglected in the analysis of these experiments. Following the quick extension there was the lengthening process which closely followed a simple exponential time course. The time constant of this exponential lengthening curve was of the same order of magnitude as that of the relaxation from contraction. Continuing from the process which gave the simple exponential curve and which was the one to which our attention was primarily directed, there might appear a very much slower extension process.

This last component did not give a strictly rectilinear record of lengthening though its curvature was often so slight as to give that appearance if only a short, late section of record was examined. The use of a long time for stabilization of the strip before beginning the experiment tended to reduce the importance of this slow component but it was at times a serious complication in the determination of asymptotes for the exponential curves studied. Although there was considerable variation in the responses of different muscle preparations and in the temperatures at which the various experiments were conducted the following statement of results

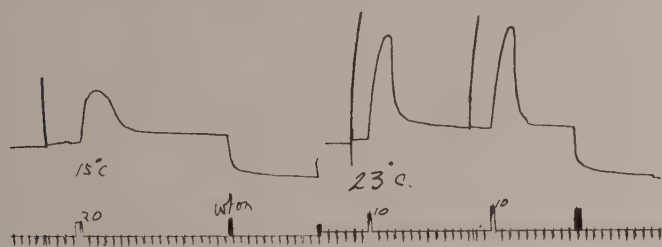


Fig. 1 Kymograph records (negatives) showing curves of contraction in response to electrical stimulation and curves of lengthening under addition of 2 gm load. Temperatures 15°C. and 23°C. Time intervals indicate 6 second intervals.

based on a particular experiment (see figures 2 and 3) is generally applicable to all the preparations used. The changes brought about by cooling the preparation from a temperature of 25°C to one of 15°C were as follows. (a) The shortening in response to a given number of shocks became less, or, the number of shocks needed to produce a given amount of shortening increased. (b) The time of rise to peak of the isotonic contraction increased. (c) The course of the isotonic relaxation became slowed (fig. 1). (d) Thus the slope of the line plotted on semilogarithmic paper became less steep (fig. 2). (e) The course of the exponential curve of lengthening following the addition of a load to the muscle showed no change. (f) The extent of the "instantaneous"

excursion probably showed no significant change. (g) The addition of atropin in concentration of 10^{-6} did not introduce any change in the above.

The nature of the records used and of the results obtained by analysis is indicated by the figures. Figure 1 reproduces (negative) prints from the kymograph records showing reactions of a muscle strip to electrical stimulation at 15°C . and at 23°C . and to the addition of a 2 gm weight at each

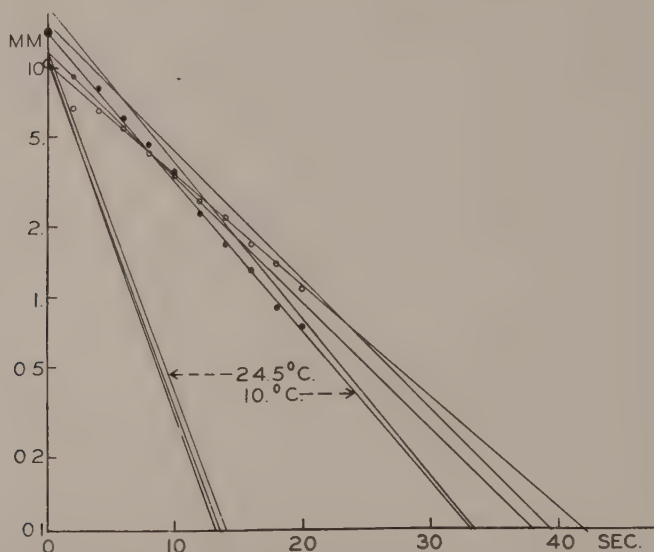


Fig. 2 Semilog plots of relaxation from contraction at high (24.5°C .) and at low (10°C .) temperatures, respectively.

of these temperatures. Time marks indicate 6 second intervals. Figures 2 and 3 plot exponential curves fitted to data obtained by measurements of records similar to those of figure 1 but from another experiment. The ruled straight lines were calculated in each case by the method of least squares. The points from which two of the lines were calculated are shown in each figure. Points for other lines were omitted in order to avoid confusion. Examination of the data by statistical methods, using the t -test for significance,

merely confirmed the conclusions made by inspection of the plotted lines. There is excellent agreement within each set of data. There is significant difference between the rates of relaxation of warm and of cold muscles. For the particular experiment taken here as example there is a significant difference between the rates of lengthening of the muscle under load and of the relaxation from contraction for either the

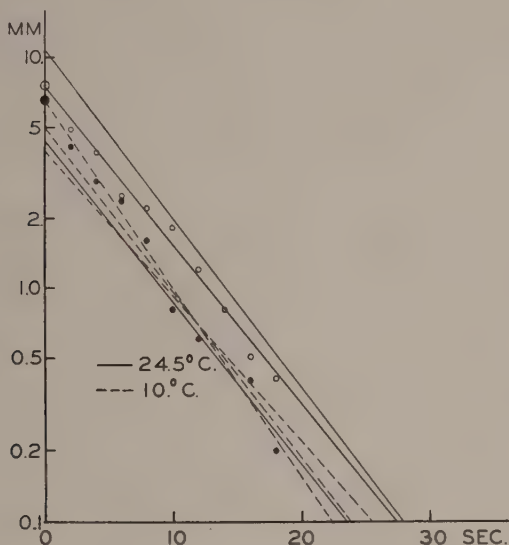


Fig. 3 Semilog plots, same scale as figure 2, of exponential curves of lengthening under addition of 2 gm load of warm and cold muscle, respectively. Same preparation as for figure 2.

warm or cold muscle. There is no significant difference between the rates of lengthening of the warm and of the cold muscles, respectively, in response to loading.

DISCUSSION AND CONCLUSIONS

The failure of the passive lengthening curve to show a significant change when the muscle is cooled stands in definite contrast to the change in the response curve following electrical stimulation. The latter shows a regular slowing both

of the contraction and relaxation phases in a manner closely resembling that of skeletal muscle similarly treated. When the exponential plots for active and passive lengthening curves are compared there will be found a close agreement at some temperature although they will be quite different at other temperatures. Such information leads to the conclusion that the similarity of the two exponentials at a given temperature is due to a coincidental and not to a causal relationship.

The interpretation placed on the results of these experiments is thus somewhat different from that used by Bozler ('51). However, in another respect it seems probable that our findings are in harmony with those of Bozler although there may appear to be confusion in the uses of the terms *active* and *passive* as employed by him and by ourselves. It is hoped that the following may eliminate any possible ambiguity. Gilson, Walker and Schoepfle ('44) showed that the time-tension curves recorded during an isometric twitch of skeletal muscle could be empirically fitted by a curve plotted from an equation involving the sum of three exponentials. Such a time-course is consistent with the hypothesis that the rise and fall of tension during the skeletal muscle twitch is dependent upon the formation of a complex which is placed at least second or third in a series of chain reactions. According to this analysis the latter part, approximately two-thirds of the lengthening, of the twitch curve is largely determined by the slowest of the three exponential processes and this part of the curve should show a nearly exponential form. The shortening and lengthening of a muscle in an isotonic contraction is believed to be governed by the same contraction process and, where acceleration effects can be neglected, to follow the same time-course. Relaxation in a muscle twitch may therefore be the expression of the course of the chemical process which brings about the opening up of acto-myosin linkages, if such explicit terminology is to be considered desirable at this time. It is to this extent and in this sense that we refer to relaxation at the ending of a twitch or tetanus as active. Since the

lengthening of a muscle in such a case is insignificant under zero load, the restoration of the muscle to its original length is dependent upon the presence of a load. We have preferred to apply the term *passive lengthening* to the lengthening which occurs when an additional load is added to the unstimulated muscle. Since Bozler ('51) considers the possibility that release of tension following a stimulated contraction of the isometric smooth muscle preparation is permitted by removal of ATP, the two hypotheses offered are not in conflict. From the difference in temperature effects reported here for rabbit stomach muscle, it appears, however, that the two processes of lengthening are not identical.

SUMMARY

Records have been made of the changes in length of strips of longitudinal muscle from the stomach of the rabbit in response to stimulation and to the addition of loads. In each case the lengthening curve is approximately that of a simple exponential. Cooling the preparation slows the relaxation from isotonic contraction but not that of the lengthening to added load. It is concluded that the two processes are not determined by the same mechanism and that the rate of relaxation from contraction is not primarily due to viscous elastic properties of the muscle but rather to the course of a definite chemical process permitting lengthening of the contractile material of the muscle under a condition of constant load.

We wish to express our very sincere thanks to Dr. R. J. Furchgott for his assistance in connection with this work.

LITERATURE CITED

- BOZLER, E. 1941 The mechanical properties of resting smooth muscle. *J. Cell. and Comp. Physiol.*, 18: 385.
 ———— 1951 Mechanism of relaxation in extracted muscle fibers. *Am. J. Physiol.*, 167: 276.

GILSON, A. S., S. M. WALKER AND G. M. SCHOEPPLE 1944 The forms of the isometric twitch and isometric tetanus curves recorded from the frog's sartorius muscle. *J. Cell. and Comp. Physiol.*, *24*: 185.

KREBS, H. A., AND K. HENSELEIT 1932 Untersuchung über die Harnstoffbildung im Tierkörper. *Ztschr. f. physiol. Chem.*, *210*: 33.

(Or see per ex., Furchgott, R. F., and M. R. Wales 1952 Utilization of compounds of Krebs cycle for contraction energy by rabbit intestinal smooth muscle. *Am. J. Physiol.*, *169*: 326).

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